Molecular epidemiology of multiresistant *Klebsiella pneumoniae* strains isolated at the Clinical Centre University of Pécs

Doctoral (PhD) dissertation

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Abbreviations

CDC	Center for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CPKP	carbapenemase producing *Klebsiella pneumoniae*
EC	epidemic clone
ECDC	European Centre for Disease Prevention and Control
ECOFF	epidemiological cut off
ECP	Epidemic Clone Pécs
EDTA	ethylene-diamine-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESBL	extended spectrum β-lactamase
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HEC	Hungarian Epidemic Clone
I	intermediate
kfu	Klebsiella Ferric iron Uptake system
LPS	lipopolysaccharide
MBL	metallo-β-lactamase
MIC	minimum inhibitory concentration
MLST	multilocus sequence typing
NBS	National Bacteriological Surveillance
NICU	neonatal intensive care unit
OMP	outer membrane protein
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
R	resistant
S	susceptible
SD	standard deviation
SDS	sodium dodecyl sulphate
ST	sequence type
TBE	Tris - boric acid – EDTA
TE	Tris – EDTA

1. Introduction

We have chosen *Klebsiella pneumoniae* to be the subject of our investigations, as this bacterium is among the most frequently encountered nosocomial pathogens, and its continuously evolving versatility and complexity of resistance mechanisms demand a particular attention [1–4].

Although national studies on the dissemination of multiresistant *K. pneumoniae* strains were conducted, and these investigations provided valuable information on a larger scale, local data are crucial for patient management and infection control.

At first I would like to give a brief overview on the species *K. pneumoniae* itself, then highlight some aspects of molecular typing methods, and finally review the literature on molecular epidemiology of multiresistant *K. pneumoniae* in Hungary.

1.1 *Klebsiella pneumoniae*

1.1.1 Taxonomy and identification

K. pneumoniae is a Gram-negative, rod-shaped, facultative anaerobic bacterium [5]. It belongs to the family Enterobacteriaceae and the genus *Klebsiella*. The genus was first described in 1885, and was named after Edwin Klebs, German pathologist. The species received the name for its ability to cause severe pneumonia.

According to recent phylogenetic analysis, the species originally defined by biochemical tests should be reconsidered as *K. pneumoniae sensu lato*, as it contains three phylogroups (KpI, KpII and KpIII) representing three different species (KpI - *K. pneumoniae sensu stricto*, KpII - *Klebsiella quasipneumoniae* and KpIII - *Klebsiella variicola*) [6–8]. The former entities *K. pneumoniae* ssp. *ozaenae*, *K. pneumoniae* ssp. *rhinoscleromatis* and *Klebsiella granulomatis* were suggested to be virulent clones of *K. pneumoniae sensu stricto* rather than distinct subspecies or species [9]. The following chapters will cover *K.*

pneumoniae ssp. *pneumoniae* as defined earlier by conventional biochemical tests.

Key characteristics for identification are fermentation of lactose, capsule production, lack of motility, no indole production, negativity in methyl red test, and typical pattern in decarboxylase assays (lysine +, arginine -, ornithine -).

It can be difficult to differentiate among *K. pneumoniae, Raoultella planticola* and *Raoultella terrigena* based on conventional biochemical tests, therefore misidentification in a clinical microbiology laboratory is possible [10, 11]. Discrimination by gene sequence analysis (16S ribosomal RNA gene sequencing or multilocus sequence typing, MLST) is yet not feasible in a clinical setting, and possible enhancements provided by the utilization of matrix-assisted laser desorption/ionisation time of flight mass spectrometry needs further evaluation [12].

1.1.2 Pathogenesis and virulence determinants

K. pneumoniae is usually considered as an opportunistic pathogen, mainly infecting hospitalised patients with underlying medical conditions. However it is capable of causing severe disease (primary liver abscess with or without metastatic complications) in otherwise healthy individuals. Such strains are often referred as being hypervirulent.

According to the molecular Koch's postulates several virulence determinants were identified in *K. pneumoniae* [13].

The polysaccharide capsule produced by the majority of *K. pneumoniae* strains is one of the main virulence determinants of this species. It interferes with phagocytosis by polymorphonuclear cells, and plays a role in resistance to serum bactericidal activity [14, 15]. At least 78 capsular serotypes (K-serotype) were defined to date [16]. The different K-serotypes seem to differ in the degree of virulence. Isolates of serotypes K1 and K2 were shown to be more virulent than non-K1/K2 isolates [17].

Hypermucoviscosity is often observed in hypervirulent strains from East-Asia. It is associated with the overproduction of the exopolysaccharide web, which is a

network of fine fibers originating from the capsular polysaccharide [18]. The hypermucoviscosus phenotype was shown to be related to various genes, including *rmpA*, *magA*, and the *cps* cluster itself [19].

The lipopolysaccharide (LPS) is an essential component of Gram-negative bacteria. Upon binding to Toll-like receptor 4 it enhances production of various proinflammatory mediators (cytokins, chemokins and major histocompatibility complex receptors). Nine different O-serogroups were identified in *K. pneumoniae* to date, of which O1 is the most prevalent among clinical isolates [20].

Resistance to complement mediated killing might be important in the development of a systemic infection. Serum resistance is attributed partly to (1) the polysaccharide capsule, (2) to the LPS and (3) to outer membrane proteins (OMPs) [21, 22].

The major adhesion factors identified in *K. pneumoniae* are type 1 and type 3 fimbriae [23, 24]. Type 1 fimbria is widely distributed among different species of Enterobacteriaceae, and is encoded by the *fim* gene cluster. It is associated with adhesion to mannose containing structures on host cells and in extracellular matrix components. Type 1 fimbria of *K. pneumoniae* is not identical with the one identified in *Escherichia coli*, but they share high degree of structural similarity. Type 1 fimbria shows phase variation: the expression is turned on during urinary tract infection, and it is turned off in the gastrointestinal tract and during lung infection. Type 1 fimbria was shown to be an important virulence factor in a murine urinary tract infection model. Type 3 fimbria is encoded by the *mrk* gene cluster, and it is involved in biofilm formation and attachment to respiratory epithel, endothel, urinary bladder cells and collagen type V. It was identified as an important colonization factor in catheter-associated urinary tract infections [25].

Biofilm formation on endotracheal tubes, intravascular and urinary catheters can serve as an infectious focus [26]. Furthermore biofilms on environmental surfaces and inside the water distributing systems might contribute to the persistence of bacteria in hospitals [27]. Inside the biofilm the activity of host defence mechanisms, antimicrobials and disinfectants is limited [28]. In addition,

exchange of the genetic material among different bacterial species within the biofilm is possible. Besides type 3 fimbria, which plays a major role, several other factors are considered to be involved in biofilm formation of *K. pneumoniae* [29–32].

Inside the blood stream and the host tissue, where the availability of iron is limited, it is crucial for the bacteria to acquire ferric ion. Various iron acquisition systems are implicated in the pathogenesis of *K. pneumoniae* infections [15]. Enterobactin is the most widely distributed siderophore among *K. pneumoniae* isolates, but its activity can be disrupted by lipocalcin-2 [33]. Other iron binding molecules, like salmochelin (a glycosylated derivative of enterobactin) and yersiniabactin, can resist binding by lipocalcin-2 [34, 35]. The later was confirmed as an important virulence determinant in pneumonia [33]. The siderophore aerobactin has lower iron affinity than enterobactin, but it is more stable and has better solubility [15]. It was shown to be a virulence factor in murine intraperitoneal and subcutan infection model. Hypervirulent *K. pneumoniae* isolates seem to produce quantitatively more siderophores, mostly aerobactin, than non-hypervirulent strains [36]. Moreover a novel pathway of iron acquisition, named *Klebsiella* Ferric ion Uptake system (kfu), was identified in hypervirulent strains [37].

In addition to the well-defined virulence factors other traits are presumed to contribute to its pathogenicity. It seems that no single virulence determinant or virulence associated trait can render a strain highly virulent or hypervirulent, rather the simultaneous expression of different factors define the virulence potential of an isolate [9, 38].

1.1.3 Clinical manifestations

K. pneumoniae infections most often occur in hospitalised patients with different underlying medical conditions, but urinary tract infection, pneumonia or primary liver abscess might develop in healthy individuals in the community.

The most common nosocomial infections by *K. pneumoniae* are urinary tract infections (cystitis, pyelonephritis, renal and perirenal abscess), pneumonia

(bronchopneumonia, lobar pneumonia, bronchitis, ventilator associated pneumonia), surgical-site infections and blood stream infections [1, 2]. Besides the aforementioned clinical presentations *K. pneumoniae* can be involved in other pulmonary (acute exacerbation of chronic obstructive pulmonary disease, empyema) indwelling medical device related (intravascular catheter, urinary catheter related), intraabdominal (liver abscess, biliary tract infections, peritonitis) and central nervous system infections (post neurosurgical meningitis, brain abscess) as well [39]. The infections can present as sporadic cases or as a part of an outbreak [40].

Pneumonia by *K. pneumoniae* is classically referred as Friedlander's disease, as it was considered to have special clinical features like: localisation to the upper lobes, fissure sign on radiography, "currant jelly" sputum, propensity to develop an abscess and frequent occurrence in alcoholic patients. Nevertheless etiologic diagnosis should not be based on the presence of these symptoms [39].

Primary liver abscess, sometimes with metastatic complications (bacteraemia, meningitis, endophthalmitis, necrotizing fasciitis), is an emerging infectious disease caused by hypervirulent strains. It is observed mainly, but not exclusively in Asia [41].

1.1.4 β-lactam resistance mechanisms

β-lactam agents include penicillins, cephalosporins, carbapenems and monobactams. These antibiotics constitute the most widely used group of antimicrobials, therefore β-lactam resistance can seriously affect patient management [42]. The most common resistance mechanism affecting β-lactam antibiotics is the production of inactivating enzymes termed as β-lactamases. Beyond their common capability of hydrolysing β-lactam antibiotics these enzymes show substantial variations in their protein structure and kinetic parameters. By utilizing these dissimilarities two β-lactamase categorization schemes were developed: (1) the Ambler (molecular) classification and (2) the Bush-Jacoby (functional) grouping [43, 44].

The Ambler classification scheme divides β-lactamases into four classes (class A-D) based on their amino-acid sequence. In contrast to class A, C and D enzymes, which have a serine molecule at their active site, class B enzymes have a divalent zinc ion, therefore they are often referred as metallo-β-lactamases.

The Bush-Jacoby classification system sorts β-lactamases into three main clusters (group 1-3) according to their substrate specificity and susceptibility to inhibitor molecules. Group 1 encloses class C β-lactamases, which preferentially hydrolyse cephalosporins and are resistant to inhibition by clavulanic acid. Group 2 is the most diverse category containing twelve subgroups. Group 2 enzymes are either class A or class D serine-β-lactamases, and their substrate specificity and inhibitor susceptibility vary according to the subgroups. Group 3 includes the class B metallo-β-lactamases, which are capable of hydrolysing carbapenems, and their specific inhibitors are chelator agents, like ethylene-diamine-tetraacetic acid (EDTA) or dipicolinic acid.

The main β-lactam resistance mechanism identified in *K. pneumoniae* will be briefly discussed hereinafter.

All *K. pneumoniae* isolates are naturally resistant to aminopenicillins (ampicillin and amoxicillin) due to the production of a chromosomally encoded group 2b SHV type β-lactamase [45]. The activity of these class A enzymes can be inhibited by clavulanic acid, sulbactam or tazobactam, hence aminopenicillin + inhibitor combinations show therapeutic effect against wild-type isolates.

The most important acquired β-lactam resistance mechanisms of *K. pneumoniae* are production of (1) extended spectrum β-lactamases (ESBL), (2) AmpC enzymes and (3) carbapenemases. All three mechanisms involve enzymes that confer cross resistance to various β-lactam compounds. Therefore isolates producing such β-lactamases are considered to be multidrug resistant, and reporting nosocomial infections caused by such isolates in Hungary is mandatory [46]. In addition multidrug resistant strains often harbour other resistance mechanism affecting non-β-lactam antibiotics as well, further shortening the therapeutic options.

Reduced susceptibility to third generation cephalosporins in *K. pneumoniae* can be attributed to ESBL and/or AmpC production.

ESBLs constitute a heterogeneous group of enzymes. No standardized definition exists, but the most often referred features are (1) the capability of hydrolysing narrow and extended spectrum cephalosporins, but (2) having no observable activity against carbapenems or cephamycins [47]. Many ESBLs belong to class A β-lactamases, thus their activity can be inhibited by clavulanic acid, sulbactam or tazobactam. This attribute is utilized for detection in routine testing [48].

Many ESBLs evolved from β-lactamases with narrower substrate spectrums through the accumulation of point mutations. The derivatives are classified into several groups according to their progenitor enzyme. The most frequently encountered groups are CTX-M, SHV and TEM. ESBLs belonging to families BEL, GES, IBC, OXA ESBL, PER, SFO, TLA and VEB can also be found in *K. pneumoniae*, but with a far lower prevalence rate [3]. The substrate specificity of ESBLs may vary slightly with respect to groups and exact location of point mutations [49].

The rate of ESBL producing isolates is continuously rising worldwide, and their spread is attributed to mobile genetic elements (plasmids, transposons, insertion sequences) and expansion of successful clones as well [3, 50].

AmpC enzymes, like ESBLs, are also a diverse group of cephalosporinases. They belong to class C, group 1 β-lactamases, and their specific inhibitor is cloxacillin. *K. pneumoniae* can acquire plasmid-borne AmpC β-lactamases (ACC, ACT, CMY, DHA, FOX, LAT, MIR, MOX), that mediate resistance to narrow and broad spectrum cephalosporins and cephamycins, but do not affect the susceptibility to carbapenems or cefepime [51]. Differences in susceptibility to β-lactamase inhibitors and resistance to cefoxitin can help to distinguish ESBLs and AmpC enzymes from each other. AmpC enzymes are less frequently responsible for third generation cephalosporin resistance in *K. pneumoniae* than ESBLs [52].

Reduced susceptibility to carbapenems in *K. pneumoniae* can be caused by (1) overproduction of either ESBL or AmpC enzymes combined with porin mutations or (2) production of carbapenemases [4, 53, 54].

β-lactam antibiotics can penetrate through two major porins in *K. pneumoniae* (OmpK35 and OmpK36) [55]. Mutations affecting their genes can alter penetration of β-lactams, including carbapenems, this way reducing susceptibility, which might be clinically relevant if it is present in conjunction with overproduction of cephalosporinases, like ESBL or AmpC [55, 56].

Carbapenemases are β-lactamases capable of hydrolysing carbapenems. Such enzymes can be found among class A (KPC, GES), class B (VIM, IMP, NDM) and class D (OXA-23-like, OXA-24-like, OXA-48-like) β-lactamases as well [4, 57]. The specific inhibitors used in routine diagnostic testing are: dipicolinic acid and EDTA for class B and boronic acid for class A carbapenemases. OXA-48-like and NDM enzymes are inhibitor resistant.

Recently a remarkable expansion of carbapenemase production by *K. pneumoniae* isolates was observed. The prevalence and type of carbapenemases vary geographically at a considerable extent [4].

The modified Hodge-test, various inhibition assays, spectrophotometric and mass spectrometric measurements are the most common phenotypic test to detect carbapenemase activity. Identification of carbapenemase genes by molecular tools are regarded as the reference method [58].

1.1.5 Resistance to other antimicrobial agents

Activity of fluoroquinolones can be diminished by accumulation of point mutations in the genes of target enzymes (*gyrAB* for DNA gyrase, *parCE* for topoisomerase IV) [59]. Besides, susceptibility to fluoroquinolones can also be reduced by (1) overproduction of the AcrAB-TolC efflux pump and by (2) plasmid mediated quinolone resistance determinants involved in the protection of target enzymes (*qnr*), active efflux (*qepA, oqxAB*) or modification of the drug (*aac(6')-Ib cr*) [60, 61]. The resistance genes *qnrA* and *qnrB* were shown to be co-localized on plasmids with other resistance genes, including ones coding for ESBLs, AmpC enzymes or carbapenemases.

Resistance to aminoglycosides relies mostly on enzymes inactivating the antibiotic through phosphorylation (APH), acetylation (AAC) or adenylation (ANT)

[62]. A bifunctional enzyme (AAC(6')-Ib-cr), capable of modifying both aminoglycosides and fluoroquinolones, can also be detected in *K. pneumoniae* [63]. Enzymes, which methylate 16S ribosomal RNA, can protect the target-site thereby conferring resistance to aminoglycosides [64].

The most common mechanism involved in chloramphenicol resistance is inactivation of the antimicrobial agent by acetylation (*cat* gene) [65].

Colistin resistance of multidrug resistant isolates is of great concern. The exact mechanism in not clear yet, but it is thought to be attributed to the modification of LPS through various pathways (*mgrB*, *phoPQ, pmr* and *ccrAB*) [66, 67]. These alterations are assumed to change the net charge of the outer membrane, resulting in reduced binding of colistin.

Concerning fosfomycin, mutations that alter the target enzymes or reduced uptake of the antibiotic are responsible for diminished susceptibility. Furthermore plasmid mediated fosfomycin inactivating enzymes (*fosAB*) can be obtained by *K. pneumoniae* isolates [68].

Overexpression or production of altered dihydropteorate synthase and/or dihydrofolate reductase enzymes can lead to resistance to sulphonamides and/or trimethoprim [69].

Resistance to tetracycline can be achieved via active efflux of the antibiotic or via protection of ribosomes [70]. Tigecycline is capable of overcoming these classical resistance mechanism, but upregulation of AcrAB efflux pumps due to overexpression of *ramA* can lead to resistance to this compound as well [71].

1.1.6 Principles of antimicrobial therapy

Infections caused by wild-type *K. pneumoniae* isolates can be treated with various antimicrobial agents. First line antibiotics include penicillin + β-lactamase inhibitor combinations (amoxicillin+clavulanic acid, ampicillin+sulbactam), first generation cephalosporins (cefalexin, cefazolin; uncomplicated urinary tract infection only), second and third generation cephalosporins, fluoroquinolones trimethoprim/sulfamethoxazole and aminoglycosides [39].

In severe infections caused by ESBL or AmpC producing isolates carbapenems are the first choice of antimicrobials [72, 73]. In order to spare carbapenems, in less severe cases, if the isolate is susceptible to the selected agent, treatment with other non-β-lactam antibiotics (fluoroquinolones, aminoglycosides, folic inhibitors, fosfomycin) or a β-lactam + β-lactamase inhibitor combination can be considered [74–76].

For infections caused by carbapenemase producing *K. pneumoniae* isolates it seems, that combination therapy could mean the optimal treatment [77]. With special considerations, the administration of carbapenems combined with other agents might be beneficial [78, 79]. Other therapeutic alternatives to be taken into account are colistin, tigecycline, fosfomycin, chloramphenicol, fluoroquinolones, and aminoglycosides.

1.1.7 Epidemiology and infection control

K. pneumoniae is widely distributed in nature: it can be found in surface water, sewage, on plants and mucosal surfaces of animals and humans [15]. The rate of gastrointestinal carriage in healthy individuals ranges between 5% and 38%. Colonization of the nasopharynx is less common in the general population (1-6%), and it is considered to be only a transient member of the skin flora.

Among hospitalized patients the rate of gastrointestinal and nasopharyngeal colonization increases proportional to the length of hospital stay, and is correlated with the administration of antibiotics [15]. Colonization bears a double burden: (1) it predisposes to infection and (2) colonized patients serve as the major reservoir for further propagation. Possible routes of transmission in a hospital setting are contaminated medical devices, hands of hospital staff and patients.

Besides colonization, which is a major risk factor (colonized patients have a four time risk over non-carriers for developing an infection), several other predisposing factors were identified: (1) impaired host defence mechanism (diabetes mellitus, alcoholism, malignancy, hepatobiliary disease, glucocorticoid therapy and renal failure), (2) prior antibiotic consumption and (3) the presence

of indwelling medical devices (urinary catheter, intravascular catheter and endotracheal tube) [15, 80].

All of the aforementioned features emphasizes, that hospitalized patients are highly vulnerable to *K. pneumoniae* infections. According to point-prevalence surveys conducted in acute-care hospitals by CDC and ECDC, *Klebsiella* spp. are etiologic agents in 11,4-11,8% of pneumonia cases; 6,0-13,6% of surgical site, 12,0-23,1% of urinary tract and 8,0-9,8% of blood stream infections. This frequency had ranked *K. pneumoniae* to be the third (CDC 2009-2010) and the fifth (ECDC, 2011-2012) among the most common pathogens in healthcare-associated infections [1, 2].

Due to the high burden of nosocomial infections, any intervention aiming the prevention of colonization and infection by *K. pneumoniae* is appreciable.

Standard hygienic measures like good hand hygiene, general cleaning, avoiding unnecessary invasive procedures, good antimicrobial prescribing practice and proper management of indwelling medical devices are essential to control the rate of healthcare-associated infections in general. In order to hamper the dissemination of multidrug resistant *K. pneumoniae* isolates (1) active screening of high-risk patients, (2) adherence to standard precautions and (3) cohort isolation of colonized/infected patients were suggested [81, 82].

1.2 Methods in molecular typing of *K. pneumoniae*

One of the main objectives of epidemiologic studies is to facilitate prevention by increasing the comprehension of factors that affect distribution, manifestation or progression of diseases. Epidemiologic analysis utilising molecular biology methods are often referred as a distinct sub discipline, named “molecular epidemiology” [83].

In the case of infectious diseases, molecular epidemiology is mostly concerned with (1) molecular fingerprinting (typing) of microbes, (2) microbial population genetics and (3) identification of factors related to hosts’ susceptibility or resistance to infection.

By typing, the dissemination of multidrug-resistant or highly virulent organisms can be examined, which can largely aid outbreak investigations and support surveillance.

The purpose of fingerprinting is to distinguish between epidemiological related and unrelated isolates. Epidemiologic connections can be presumed by the confirmation of (1) a common ancestor (vertical dissemination, clonal expansion) or (2) a common mobile genetic element (horizontal dissemination). Such relatedness can verify a common source for infections, elucidate the route of transmission or prove recurrence.

The application of molecular tools in typing has resulted in a high gain of discriminatory power as compared to conventional methods. Today's most widely used molecular typing techniques for *K. pneumoniae* include macrorestriction profile analysis by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) [84].

In the near future – with the increasing accessibility to whole-genome sequencing – molecular epidemiology might evolve into "genomic epidemiology" reaching the end-point in resolution, and accomplishing real-time outbreak investigation [85].

1.2.1 Macrorestriction profile analysis by pulsed-field gel electrophoresis

PFGE is a special electrophoretic method used to separate large DNA fragments of bacteria after treatment with a specific restriction endonuclease (*Xba*I in the case of *K. pneumoniae*) [86]. The term "macrorestriction profile" refers to the fragmentation pattern revealed by PFGE. By comparing such profiles, the relatedness of isolates can be assessed.

In brief this method consists of the following steps:

- *Casting of agarose plugs*
  Agarose plugs are casted from a mixture of bacterial suspensions and molten agarose. All further reactions (lysis of the cells, restriction digestion) will take place inside these plugs thereby preventing

premature and unspecific fragmentation of the chromosome during the entire process.

- *Lysis of cells*

  This phase is done by incubating the agarose plugs in a lysis buffer containing proteinase K. By lysing the bacterial cell wall the intact chromosome is released.

- *Washing of plugs*

  Washing is required to eliminate the lysis buffer and thereby to prepare the plugs for digestion.

- *Restriction digestion*

  After washing the plugs are incubated in a reaction buffer containing restriction endonuclease *Xba*I. The endonuclease fragments the DNA with cutting it in several specific locations recognised by the enzyme. The size of the fragments depends on the position of the recognition sites.

- *Pulsed-field gel electrophoresis*

  After digestion the plugs are placed into the wells of an agarose gel so that electrophoretic separation of the DNA fragments can be initiated. As compared to conventional agarose gel electrophoresis, which utilizes an unidirectional current, in PFGE the electric field is continuously alternating among several pairs of electrodes. The position of the electrodes and the equal switch times for each direction will ensure a net forward migration and adequate separation of large DNA fragments. After electrophoresis the gel should be stained with ethidium bromide so that the fragments can be visualized under ultraviolet light.

- *Evaluation*

  Finally, the resulting patterns should be compared to each other in order to ascertain the relatedness of the isolates. The comparison is most often performed by computer softwares, but supervision by a skilled expert is mandatory.

Macrorestriction profile analysis by PFGE is most useful in local outbreak investigations, because it has relatively high discriminatory power, and the intralaboratory reproducibility of results is good, but interlaboratory comparisons can be problematic.

1.2.2 Multilocus sequence typing

MLST is the choice for studies with timely and spatially more distant isolates. For discrimination, MLST utilizes DNA sequence data of internal regions of several housekeeping genes. In the case of *K. pneumoniae rpoB* (β-subunit of RNA polymerase), *gapA* (glyceraldehyde 3-phosphate dehydrogenase), *mdh* (malate dehydrogenase), *pgi* (phosphoglucose isomerase), *phoE* (phosphorine E), *infB* (translation initiation factor 2) and *tonB* (periplasmic energy transducer) genes are investigated [87]. A separate allele number is assigned to each unique sequence, and the sequence type (ST) is determined by the combination of allele numbers of these seven genes. Each sequence type corresponds to just one exact combination of alleles. Every novel sequence type and allele number should be deposited in the public database, this way international comparison with a broad collection of isolates is possible.

Because of highly conserved nature of the housekeeping genes investigated, the resulting sequence types are highly stable over time, but this feature generates some lack in resolution at the same time. Due to great stability, standardization and publicly available databases it is very efficient in large scale, international studies.

1.3 Multiresistant *K. pneumoniae* in Hungary

During the last two decades several articles on the molecular epidemiology of multiresistant *K. pneumoniae* in Hungary were published. For better transparency these studies are listed chronologically in Table 1. The data summarized in the table are the year and region of isolation, the β-lactamases

identified (with special focus on ESBLs and carbapenemases) and the clonality detected (with designation of clones where available).

According to Table 1 the delimitation of the following three time periods seems reasonable: (1) ESBL production in 1996-2002, (2) ESBL production in 2003-2008 and (3) carbapenemase production from 2008.

1.3.1 ESBL production (1996-2002)

This initial period was governed by SHV type ESBLs.

The first ESBLs in Hungarian *K. pneumoniae* isolates were detected in 1996, and their genes were identified as *bla*$_{\text{SHV-2}}$ and *bla*$_{\text{SHV-5}}$ [89].

Later SHV-2a and SHV-5 became the dominant ESBL types, and the geographic distribution of these enzymes showed marked differences [92]. SHV-5 was found to be universally disseminated, while SHV-2a was confined to the southern and eastern part of the country.

During this period several nosocomial outbreaks due to ESBL producing isolates occurred in separate neonatal intensive care units (NICUs) across the country [90, 91, 93]. The raised occurrence was attributed to the dissemination of epidemic resistance plasmids harbouring either *bla*$_{\text{SHV-5}}$ or *bla*$_{\text{SHV-2a}}$.

1.3.2 ESBL production (2003-2008)

In 2003 when CTX-M type enzymes were first identified in Hungary, the epidemiologic scene started to change [94]. The CTX-M positive isolates at that time belonged to a common pulsotype, harboured the gene *bla*$_{\text{CTX-M-15,}}$ were highly resistant to ciprofloxacin, and originated from different geographical regions of the country. The name Hungarian Epidemic Clone (HEC) was proposed for the strains belonging to this novel pulsotype.

By 2005 HEC became predominant alongside with two other ciprofloxacin resistant, CTX-M-15 producing clones (Epidemic Clone II, EC II; and Epidemic Clone III, EC III) [50]. According to MLST the clones were identified as ST15 (HEC), ST147 (EC II) and ST11 (EC III).

1. Table Studies on molecular epidemiology of multiresistant *K. pneumoniae* isolates in Hungary (HEC = Hungarian Epidemic Clone; EC = Epidemic Clone; NICU = neonatal intensive care unit)

Year	Region	β-lactamase	Clonality	Ref.
1995-1996	Debrecen	putative overproduction of chromosomal enzyme	not analysed	[88]
1996	nationwide	SHV-2	not analysed	[89]
		SHV-5		
1998	Szolnok (NICU)	SHV-5	polyclonal	[90]
2001-2005	Budapest (NICU)	SHV-5	polyclonal	[91]
2002-2003	nationwide	SHV-2a	polyclonal	[92]
		SHV-5		
2002-2003	nationwide (NICU)	SHV-2a	polyclonal	[93]
		SHV-5		
2003	nationwide	CTX-M-15	HEC	[94]
2005	nationwide	CTX-M-15	HEC/ST15	[50]
			EC II/ST147	
			EC III/ST11	
2005-2008	nationwide	SHV ESBL	polyclonal	[95]
		CTX-M	HEC/ST15	
			EC II/ST147	
2006	Budapest	SHV-2	not analysed	[96]
		SHV-5		
		SHV-12		
		CTX-M-15		
2006-2008	nationwide	SHV-2a	EC IV/ST274	[97]
		CTX-M-15		
2008-2009	Miskolc	SHV-12, KPC-2	ST258	[98]
2009	Budapest	CTX-M-15, VIM-4	EC III/ST11	[99]
2012	Szeged	CTX-M-15, OXA-162	ST15	[100]

Besides the ongoing dissemination of CTX-M-15 producing epidemic clones in the adult care setting, outbreaks on NICUs remained to be solely caused by SHV type ESBLs [95].

This duality was further emphasized with the emergence of a fourth epidemic clone (EC IV/ST274) in 2006 [97]. Isolates of EC IV were shown to produce either SHV-2a or CTX-M-15. The gene *bla*$_{SHV2a}$ was located on a plasmid with identical restriction profile to the one identified during the neonatal nosocomial outbreak in 1998. The restriction profile of the plasmid harbouring the *bla*$_{CTX-M-15}$ gene matched the one of HEC from 2005. The SHV-2a producing strains were isolated exclusively from NICUs, while the CTX-M-15 producing ones originated solely from adult healthcare departments.

The spectrum of ESBLs present in *K. pneumoniae* isolates in Hungary was further broadened by the detection of *bla*$_{SHV-12}$ in 2006 [96].

1.3.3 Carbapenemase production (2008-)

Since 2008 carbapenemase producing isolates have been detected across the country in an increasing number. Regarding β-lactamase composition and clonality, a considerable versatility could be observed. Three distinct classes of carbapenemases (class A – KPC, class B – VIM, class D – OXA-162) in conjunction with the presence of different ESBL types and occurrence in different high risk clones were detected in geographically distant parts of the country [98–100].

The ST258 isolates from the north-eastern Hungarian outbreak in 2008-2009 presented with an extensively drug resistant phenotype owing to the combination of (1) KPC-2 carbapenemase production, (2) SHV-12 ESBL production and (3) resistance to colistin [98]. In the case of KPC-2, importation from abroad could be suggested, as the index patient of the outbreak was directly transferred from a Greek hospital to a Hungarian healthcare institute.

Acquisition of *bla*$_{VIM-4}$ by the CTX-M-15 producing EC III/ST11 was observed in the capital city in 2009 [99]. The carbapenemase gene was found to be located

on an integron (In238b), which was identical to the one originally described in *Pseudomonas aeruginosa* in 2002 [101].

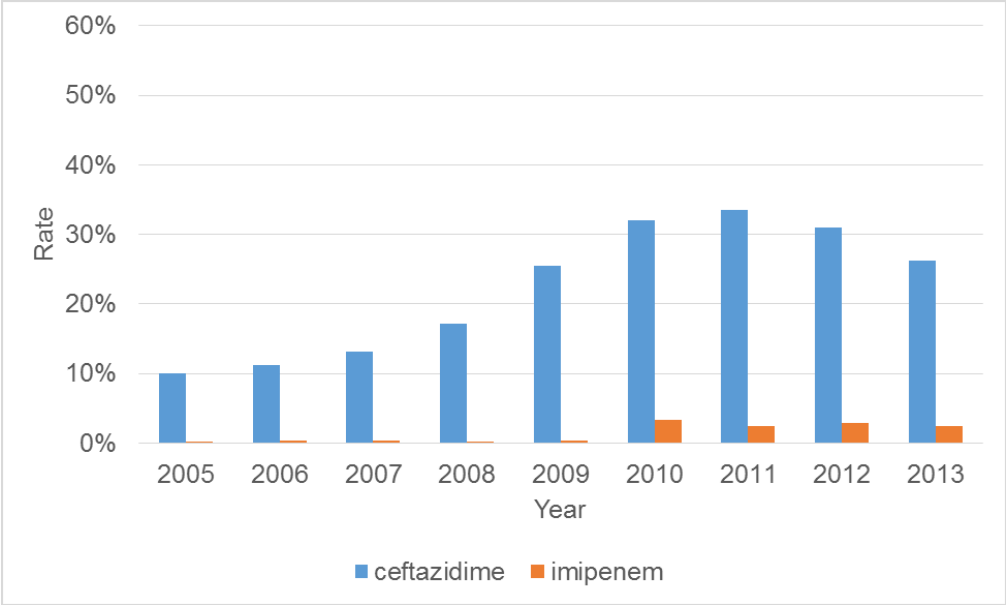
The third type of carbapenemase, namely OXA-162, was detected in south-eastern Hungary in 2012 [100]. The isolates were proven to belong to ST15, and beyond carbapenemase production they expressed *bla*$_{\text{CTX-M-15}}$ as well.

1.3.4 Data from the National Bacteriological Surveillance

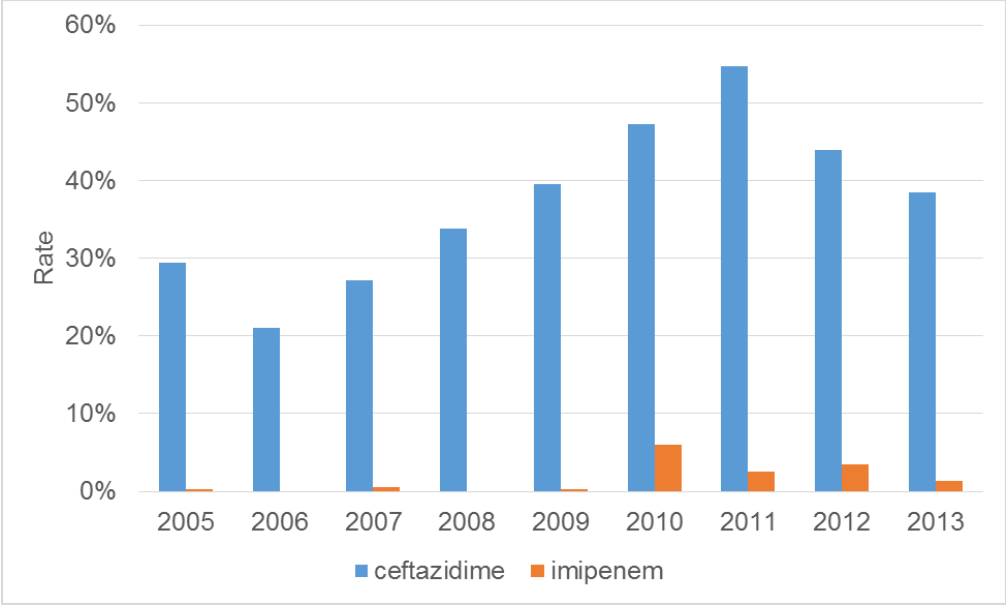
The National Bacteriological Surveillance (NBS) database contains the annual rate of resistant, intermediately susceptible and susceptible isolates for the most important species and antibiotic groups as totalized from 32 collaborating laboratories [102]. This databank largely enlightens the observation of trends in resistance.

In order to approximate the extent of ESBL and carbapenemase production by *K. pneumoniae* in Hungary the rate of non-susceptibility to ceftazidime (for ESBL estimation) and imipenem (for carbapenemase estimation) as appeared in the National Bacteriological Surveillance database are presented in Figures 1 and 2. These data cannot represent the true rate of ESBL or carbapenemase production because: (1) resistance to ceftazidime/imipenem can be attributed to other mechanisms as well; and (2) not all ESBL/carbapenemase genes confer clinically relevant resistance to these compounds. However changes in the prevalence of ESBL/carbapenemase production are assumed to be reflected in the rate of non-susceptibility to ceftazidime/imipenem at a substantial degree as resistance to these compounds is mostly attributed to those mechanisms.

According to data calculated for all clinical samples (Figure 1) the degree of non-susceptibility to ceftazidime increased considerably between 2005 and 2013. The rates varied from 10.0% in 2005 to 33.5% in 2011. The significance of the apparent decline starting in 2012 should be judged in the future. Highest rate for non-susceptibility to imipenem was observed in 2010 (3.4%), and since than the rate fluctuated around 2.3-2.9%.



1. Figure Non-susceptibility of *K. pneumoniae* isolates to ceftriaxone and imipenem for all clinical samples according to National Bacteriological Surveillance



2. Figure Non-susceptibility of *K. pneumoniae* isolates to ceftriaxone and imipenem for samples from invasive infections according to National Bacteriological Surveillance

Data based on blood culture and cerebrospinal fluid samples (Figure 2) indicates the rate of non-susceptibility observed for serious, invasive infections. The dynamics of alterations is similar to the ones observed for all clinical samples, but the rates are much higher (21.0-54.7% for ceftriaxone, 0.0-6.0% for imipenem).

In the view of these numbers a significant burden on the Hungarian healthcare system by multidrug resistant *K. pneumoniae* isolates can be presumed.

1.3.5 Data from the National Nosocomial Surveillance System

Reporting of nosocomial infections and outbreaks caused by multidrug resistant pathogens, including ESBL, AmpC and carbapenemase producing or carbapenem non-susceptible *K. pneumoniae* isolates, is mandatory in Hungary.

According to the reports from the National Nosocomial Surveillance System three to eight nosocomial outbreaks involving ESBL producing *K. pneumoniae* isolates were registered annually between 2007 and 2011 [46].

When considering nosocomial infections attributed to multidrug resistant pathogens between 2007 and 2010, *K. pneumoniae* was the second most common causative agent, right after methicillin resistant *Staphylococcus aureus*. In 2011 multiresistant *K. pneumoniae* slipped back to third place due to the increasing prevalence of multidrug resistant *Acinetobacter baumannii* [46].

2. Aims

As no extensive studies had been performed on multiresistant *K. pneumoniae* isolates from the Clinical Centre University of Pécs before we started our work, the aim of our investigations was to gain comprehensive knowledge on the isolates with acquired β-lactam resistance mechanism(s) in order to (1) estimate the dissemination of specific multiresistant clones in time and place, (2) look for clonal characteristics in antimicrobial susceptibility pattern and virulence associated factor content, and (3) support an ongoing surveillance by acquiring a well-characterised strain collection.

The following initial questions were framed:

- How did the rate of β-lactam resistance in *K. pneumoniae* isolates change over time in the Clinical Centre University of Pécs?

- How do our rates compare to national data?

In order to investigate whether the changes in β-lactam resistance rates could be explained by the dissemination of specific clones or resistant plasmids two separate studies were designed. The first study assessed the early period of ESBL production (2004-2008), while the second study was designed to investigate the ongoing dissemination of carbapenemase producing *K. pneumoniae* (CPKP) isolates in our institution (2009-2011). For these studies the subsequent specific questions were raised:

Study 1 (ESBL 2004-2008)

- Which ESBL genes were the most prevalent?

- Which ESBL producing clones were the most prevalent?

- How were the different clones distributed in time and place?

- What kind of resistance patterns were characteristic for the different clones?

- What was the distribution of various virulence associated traits among different clones?

Study 2 (CPKP 2009-2011)

- What was the molecular background of carbapenemase production?

- Were the isolates clonally related?

- What antibiotics were the isolates susceptible to?

For better clarity, the discussion of the three different topics are sorted under three separate headings of the thesis according to the grouping of the questions above.

3. Trends in β-lactam resistance of *K. pneumoniae* isolates

3.1 Materials and methods

In order to estimate trends in β-lactam resistance of *K. pneumoniae* isolates in the Clinical Centre University of Pécs, routine data from the laboratory information system (Medbakter) were collected and analysed on a yearly basis for the time period 2003-2014. The investigation was performed with respect to recommendations by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) [103]. Duplicate isolates were excluded according to susceptibility pattern with the following considerations:

- two isolates of the same patient were considered to be different if they showed a major difference (susceptible → resistant, resistant → susceptible) in the markers stated below;
- two isolates of the same patient were considered to be identical if they did not show major differences in the markers, consequently the duplicate isolate was eliminated from the study;
- minor differences (susceptible → intermediate, intermediate → susceptible, intermediate → resistant, resistant → intermediate) were omitted since they were regarded as variations in phenotypic expression.

The markers were chosen to be the pool of susceptibility results of several antimicrobials plus the absence/presence of enzyme production, namely:

- marker “3CEF”: cefotaxime, ceftazidime, ceftriaxone or ESBL/AmpC production (ESBL/AmpC negative → susceptible, ESBL/AmpC positive → resistant)
- marker “CARB”: ertapenem, imipenem, meropenem or carbapenemase production (carbapenemase negative → susceptible, carbapenemase positive → resistant).

If discrepancies were noted within the pool (for example ertapenem resistant and meropenem susceptible) only the more resistant phenotype was taken into consideration.

The pooling of several agents and enzyme production seemed reasonable as (1) not all antibiotics were tested for all isolates, (2) enzyme production was not always designated, and (3) the changing from CLSI (Clinical and Laboratory Standards Institute) to EUCAST (European Committee on Antimicrobial Susceptibility Testing) methodology in the beginning of 2014 affected reporting of the results.
Finally, six data series were calculated:

(1) *ceph*$_{all}$: rate of *K. pneumoniae* isolates resistant to third generation cephalosporins and/or demonstration of either ESBL or AmpC production considering all clinical samples

$$ceph_{all} = \frac{\text{number of isolates with resistance to "3CEF" for all samples}}{\text{total number of isolates for all samples}}$$

(2) *carb*$_{all}$: rate of *K. pneumoniae* isolates resistant to carbapenems and/or demonstration of carbapenemase production considering all clinical samples

$$carb_{all} = \frac{\text{number of isolates with resistance to "CARB" for all samples}}{\text{total number of isolates for all samples}}$$

(3) *ceph*$_{inv}$: rate of *K. pneumoniae* isolates resistant to third generation cephalosporins and/or demonstration of either ESBL or AmpC production considering samples from invasive infections (blood, cerebrospinal fluid) only

$$ceph_{inv} = \\ = \frac{\text{number of isolates with resistance to "3CEF" for samples from invasive infections}}{\text{total number of isolates for samples from invasive infections}}$$

(4) $carb_{inv}$: rate of *K. pneumoniae* isolates resistant to carbapenems and/or demonstration of carbapenemase production considering samples from invasive infections (blood, cerebrospinal fluid) only

$$carb_{inv} = \\ = \frac{\text{number of isolates with resistance to "CARB" for samples from invasive infections}}{\text{total number of isolates for samples from invasive infections}}$$

(5) wt_{inc}: incidence density of *K. pneumoniae* isolates without acquired β-lactam resistance mechanism for all clinical samples

$$wt_{inc} = \frac{\text{number of isolates susceptible to "3CEF" and "CARB"}}{\text{number of days of hospitalisation}}$$

(6) mdr_{inc}: incidence density of *K. pneumoniae* isolates with acquired β-lactam resistance mechanism for all clinical samples

$$mdr_{inc} = \frac{\text{number of isolates resistant to "3CEF" or "CARB"}}{\text{number of days of hospitalisation}}$$

Number of days of hospitalization for Clinical Centre University of Pécs was obtained from the database of National Health Insurance Fund of Hungary (www.gyogyinfok.hu).

3.2 Results

With the exclusion of duplicate isolates, altogether 8535 *K. pneumoniae* isolates were registered in Medbakter between 1 January 2003 and 31 December 2014. Of these 1889 (22.1%) were resistant to “3CEF”, and 433 (5.1%) were resistant to “CARB”. Of the latter all but one isolate exhibited resistance to the two markers simultaneously.

Therefore it can be concluded that:

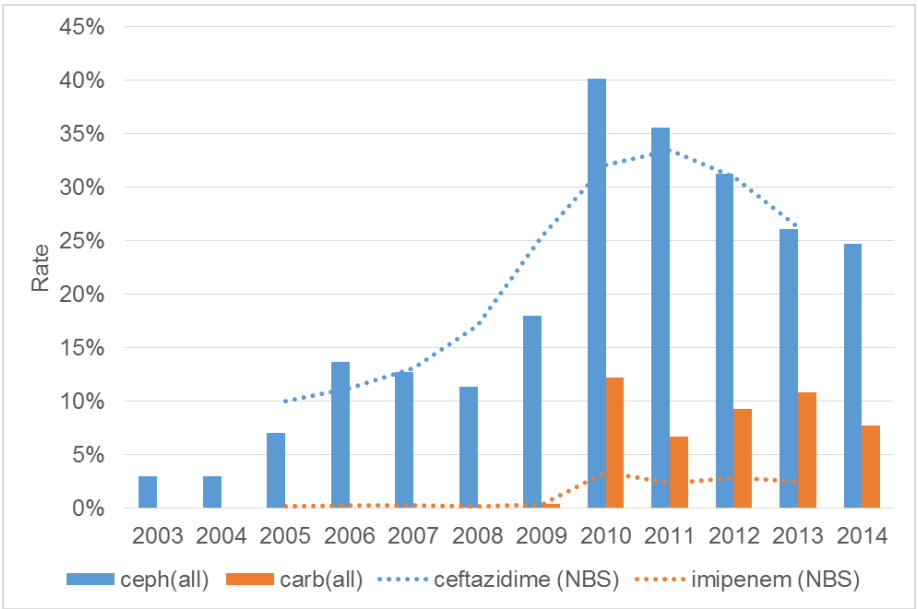
- 6645 (77.9%) isolates showed no sign for acquired β-lactam resistance mechanisms;
- 1457 (17.1%) isolates were resistant to third generation cephalosporins and/or produced ESBL/AmpC, but were susceptible to carbapenems, and produced no carbapenemases;
- 432 (5.1%) isolates were resistant to third generation cephalosporins and carbapenems simultaneously or were resistant to third generation cephalosporins and produced carbapenemase concurrently;
- 1 (<0.1%) isolate was resistant to carbapenems, but was susceptible to third generation cephalosporins.

The most frequent source of multiresistant isolates were Internal Medicine 1 (28.3%), Urology (26.8%), Internal Medicine 2 (12.8%), Surgery (11.5%), Anaesthesia and Intensive Therapy (8.4%) and Neurology (3.0%).

The annual rates of resistance ($ceph_{all}$, $carb_{all}$, $ceph_{inv}$, $carb_{inv}$) are presented in Tables 2 & 3 and Figures 3 & 4. The corresponding data on non-susceptibility rates to ceftazidime and imipenem from the National Bacteriological Surveillance (NBS) database is also featured in order to enlighten the comparison. The calculated annual incidence densities are shown in Table 4 and Figure 5.

2. Table Annual rate of multiresistant *K. pneumoniae* isolates at the Clinical Centre University of Pécs and corresponding data from NBS for all clinical samples

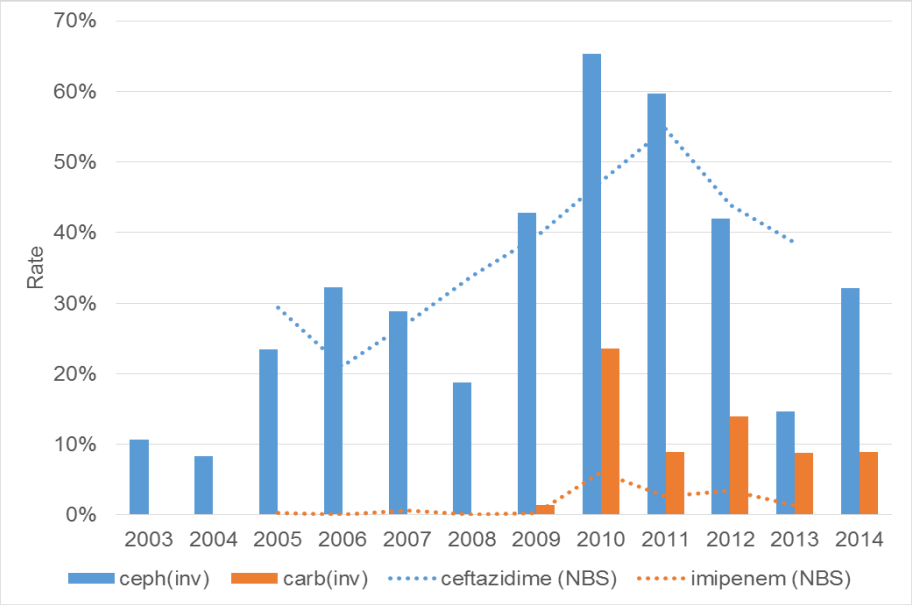
Year	*ceph*$_{all}$	ceftazidime (NBS)	*carb*$_{all}$	imipenem (NBS)
2003	3.0%	NA	0.0%	NA
2004	3.0%	NA	0.0%	NA
2005	7.0%	10.0%	0.0%	0.2%
2006	13.7%	11.2%	0.0%	0.3%
2007	12.8%	13.1%	0.0%	0.3%
2008	11.4%	17.1%	0.0%	0.2%
2009	18.0%	25.5%	0.4%	0.4%
2010	40.1%	32.1%	12.2%	3.4%
2011	35.6%	33.5%	6.7%	2.4%
2012	31.3%	31.0%	9.3%	2.9%
2013	26.1%	26.3%	10.9%	2.5%
2014	24.7%	NA	7.8%	NA



3. Figure Annual rate of multiresistant *K. pneumoniae* isolates at the Clinical Centre University of Pécs and corresponding data from NBS for all samples

3. Table Annual rate of multiresistant *K. pneumoniae* isolates at the Clinical Centre University of Pécs and corresponding data from NBS for samples from invasive infections

Year	*ceph*$_{inv}$	ceftazidime (NBS)	*carb*$_{inv}$	imipenem (NBS)
2003	10.6%	NA	0.0%	NA
2004	8.3%	NA	0.0%	NA
2005	23.4%	29.4%	0.0%	0.3%
2006	32.3%	21.0%	0.0%	0.0%
2007	28.9%	27.1%	0.0%	0.6%
2008	18.8%	33.8%	0.0%	0.0%
2009	42.9%	39.5%	1.4%	0.3%
2010	65.4%	47.2%	23.6%	6.0%
2011	59.7%	54.7%	9.0%	2.6%
2012	42.0%	43.9%	14.0%	3.5%
2013	14.7%	38.5%	8.8%	1.3%
2014	32.1%	NA	8.9%	NA



4. Figure Annual rate of multiresistant *K. pneumoniae* isolates at the Clinical Centre University of Pécs and corresponding data from NBS for samples from invasive infections

4. Table Incidence density of *K. pneumoniae* isolates at the Clinical Centre University of Pécs without (wt_{inc}) or with (mdr_{inc}) acquired β-lactam resistance mechanisms

Year	wt_{inc} /1000 patient days	mdr_{inc} /1000 patient days
2003	1.26	0.04
2004	1.30	0.04
2005	1.14	0.09
2006	1.31	0.21
2007	1.45	0.21
2008	1.64	0.21
2009	1.69	0.37
2010	1.66	1.11
2011	1.31	0.72
2012	1.36	0.62
2013	1.31	0.46

5. Figure Incidence density of *K. pneumoniae* isolates at the Clinical Centre University of Pécs without (wt_{inc}) or with (mdr_{inc}) acquired β-lactam resistance mechanisms

3.3 Discussion

With the utilization of markers “3CEF” and “CARB” tracking of the following β-lactam resistance mechanisms became possible:

- those affecting third generation cephalosporin susceptibility, namely ESBL and AmpC (*ceph*$_{all}$, *ceph*$_{inv}$);
- and those affecting carbapenem susceptibility, namely carbapenemase production and hyper production of ESBL/AmpC in conjunction with porin mutations (*carb*$_{all}$, *carb*$_{inv}$).

All four data series showed considerable changes in time. When looking at Figure 3 two extensive escalations in rates can be recognised: (1) the first one occurred between 2004 and 2006 (3.0-13.7% for *ceph*$_{all}$) and (2) the second happened between 2008 and 2010 (11.4%-40.1% for *ceph*$_{all}$ and 0.0-12.2% for *carb*$_{all}$). Both escalations were followed by a slight decrease in resistance rates.

The first increment in *ceph*$_{all}$ in 2004-2006 was solely attributed to an increase in ESBL production, because acquired AmpC production was not identified at that time, and it is still detected rarely in *K. pneumoniae* in our institution (data not shown).

For the increment in *carb*$_{all}$ between 2008 and 2010 carbapenemase production was suggested as the main underlying mechanism, as since the first detection of carbapenemase production by *K. pneumoniae* in our institution in November 2009, all *K. pneumoniae* isolates with reduced susceptibility to carbapenems were consistently positive in the modified Hodge-test presuming the production of carbapenemases.

As comparing *ceph*$_{all}$ and *carb*$_{all}$ to rates by National Bacteriological Surveillance, it can be concluded that the general trends observed for local and national data were much alike, but two major differences noticed in the scale of the numbers should be pointed out. First, in 2010 ceph$_{all}$ and carb$_{all}$ significantly exceeded the corresponding national rates of non-susceptibility to ceftazidime and imipenem, and second, *carb*$_{all}$ remained at a substantially higher level even after 2010 (it seemed to fluctuate around 8.7% as compared to national data with an average of 2.6%). Both remarks might be explained by the dissemination of

carbapenemase producers. Considering that the majority (>99%) of the isolates counted as resistant to “CARB” produced carbapenemase and ESBL simultaneously (see section 4.3 for details), it can be concluded that the increased $ceph_{all}$ rate in 2010 was mainly attributable to the surplus generated by the emergence of carbapenemase producing isolates in the Clinical Centre University of Pécs. The second remark (higher level of $carb_{all}$ after 2010 than the national rate of non-susceptibility to imipenem) can also be primarily credited to the expansion of carbapenemase producers locally, but also the differences in the calculation of rates might have caused a bias towards higher local percentages, since $carb_{all}$ shows not only the carbapenem non susceptible isolates (as data by National Bacteriological Surveillance), but also those isolates that are carbapenem susceptible despite the production of a carbapenemase.

The rates calculated for samples from invasive infections ($ceph_{inv}$ and $carb_{inv}$) showed similar dynamics to rates determined for all samples ($ceph_{all}$, $carb_{all}$), but the scale and fluctuations were higher. The lower rates for all samples can be explained by the fact that this group includes many isolates from outpatients, where resistance rates are known to be lower. The greater fluctuations for samples from invasive infections might be attributed to the smaller sample size.

For $ceph_{inv}$, $carb_{inv}$ and the corresponding national data, it can be affirmed that they resemble the procedures observed for all samples, but the lower number of isolates and consequent higher fluctuations affect evaluation and comparison. Therefore trends described for all samples should be acknowledged for serious invasive infections as well.

The mdr_{inc} and wt_{inc} data series show the number of novel infections and colonisations by isolates with or without acquired β-lactam resistance mechanism per 1000 patient days, respectively. The incidence density for isolates without acquired β-lactam resistance mechanism (wt_{inc}) seemed to vary around 1.4 / 1000 patient days throughout the study period (Figure 5). This suggest that infections and colonisations by multiresistant *K. pneumoniae* isolates with acquired β-lactam resistance mechanisms happened in addition to infections and colonisations by isolates without acquired β-lactam resistance mechanism, imposing a surplus burden on patients.

4. Molecular epidemiology of multiresistant *K. pneumoniae* isolates

4.1 Materials and methods

In order to investigate the underlying factors of the two escalations observed in β-lactam resistance rates (for $ceph_{all}$ in 2004-2006 and for $carb_{all}$ between 2008 and 2010), two separate studies were conducted. The first one (study 1: ESBL 2004-2008) was aimed to examine the propagation of ESBL producing isolates between 2004 and 2008. The purpose of the second one (study 2 CPKP 2009-2011) was to investigate the dissemination of carbapenemase producing *K. pneumoniae* (CPKP) isolates.

As many of the methods overlap for the two studies, I found that the compounding of this section would be more convenient. Any differences in the procedures are indicated.

4.1.1 Isolates

For the first study (ESBL 2004-2008) 102 ESBL producing *K. pneumoniae* isolates from 2004-2008 were selected. For the second study (CPKP 2009-2011) 102 carbapenemase producing *K. pneumoniae* isolates from 2009-2011 were chosen.

The selections were performed so as to represent the study periods, regarding time of collection and departments (Table 5). Only one isolate per patient was included. The sources of specimens are presented in Table 6.

Identification of species was performed with standard biochemical procedures (lactose fermentation, Nógrády's polytropic medium, motility, indole, urease, citrate, methyl red, lysine decarboxylase, arginine dihydrolase and ornithine decarboxylase tests) [5].

5. Table Source of isolates present in the studies

Department	**Study 1** ESBL 2004-2008	**Study 2** CPKP 2009-2011
Anaesthesia and Intensive Therapy	11	8
Internal Medicine 1	40	35
Internal Medicine 2	5	15
Neurology	3	4
Surgery	3	9
Urology	35	23
Other	5	8
Total	**102**	**102**

6. Table Distribution of isolates with respect to clinical specimens

Clinical specimen	**Study 1** ESBL 2004-2008	**Study 2** CPKP 2009-2011
urine	57	70
blood	22	12
lower respiratory tract	7	5
wound, pus or aspirate	10	7
vascular catheter	3	2
other	3	6
Total	**102**	**102**

4.1.2 Molecular typing

For molecular typing the combination of PFGE and MLST was chosen in order to gain the high discriminatory power by PFGE and to achieve the ability of interlaboratory comparison by MLST.

Macrorestriction profile analysis by PFGE was performed according to the standardized Pulsenet protocol [86]. Overnight bacterial cultures (Luria agar, 37°C) were suspended in Cell Suspension Buffer (100 mM Tris, 100 mM EDTA, pH 8.0), and the optical density of the suspension was adjusted to OD=1.4 at 600 nm. Then 120 µl molten 1.2 % agarose for PFGE (Sigma-Aldrich, St. Louis, MO, USA) in proteinase K/Cell Lysis Buffer (1 mg/mL proteinase K, 50 mM Tris: 50 mM EDTA, 1% Sarcosyl) was added to 120 µl bacterial suspension. The mixture was dispensed into plug molds. After solidification the agarose plugs were transferred into 2 ml proteinase K/Cell Lysis Buffer (0.1 mg/mL proteinase K, 50 mM Tris: 50 mM EDTA, 1% Sarcosyl) and were incubated at 50°C at 150 rpm agitation for 3 hours. The plugs were washed once with ultrapure water, twice with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and twice with 33mM Tris buffer (pH 7.9). All washing steps were performed at 50°C. Finally plugs were moved to 50 µl 1x restriction reaction buffer. After incubation for 30 minutes at room temperature, the buffer was removed, and 50 µl restriction enzyme mix (0.6 U/µl *XbaI* in 1x reaction buffer) was added. The plugs were incubated at 37°C for 4 hours. Then the restriction enzyme mix was discarded, and plugs were incubated in 0.5x TBE puffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA) with 2 mM thiourea for 15 minutes. Finally the plugs were loaded into the wells of a freshly casted agarose gel (1.2% agarose for PFGE in 0,5x TBE buffer with 2 mM thiourea). The wells were sealed with 1.2% agarose. PFGE was run for 24 hours on Gene Navigator System (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with parameters presented in Table 7. The gel was stained with ethidium-bromide for 3 hours. The resulting macrorestrictional profiles were evaluated with softwares Bio-Capt (Vilber Lourmat, Marne-la-Vallée, France) and Fingerprint II (Bio-Rad, Hercules, CA, USA). Similarity indexes were calculated by Dice-coefficient. The unweighted pair group method with arithmetic means method

was used for clustering. Clones were defined as group of isolates with > 85% similar patterns. For internal control and molecular weight standard *Salmonella enterica* serotype Braenderup provided by National Center for Epidemiology was used.

For study 1 (ESBL 2004-2008) MLST Protocol 2 (with universal sequencing primers) of Pasteur Institute (http://bigsdb.web.pasteur.fr/klebsiella/) was performed for single representatives of major ESBL clones and SHV-5 ESBL producing minor clones [87]. The internal regions of the seven housekeeping genes were amplified using universal primer pairs, KlenTaq polymerase (Sigma-Aldrich, St. Louis, MO, USA) and conditions described in the protocol. The electrophoretic bands were cut from the agarose gel, and purified with QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). The purified template DNAs were sent to a collaborating laboratory in Vienna for sequencing.

For study 2 (CPKP 2009-2011) MLST was performed for three selected isolates at the National Center for Epidemiology (Budapest, Hungary) [99].

7. Table Parameter settings used for PFGE

	Pulsations	**Switch time for N-S**	**Switch time for E-W**	**Running time**
Phase 1	3554	0,5 sec	0,5 sec	20 hours
Phase 2	228	40 sec	40 sec	3 hours
Phase 3	66	54 sec	54 sec	1 hour
Voltage		**Current**		**Power**
180 V		300 mA		1 W

4.1.3 Detection of β-lactamases

Production of ESBL was determined with the combined disc method using ceftazidime + ceftazidime-clavulanic acid and cefotaxime + cefotaxime-clavulanic acid discs (Bio-Rad, Hercules, CA, USA) [104]. In brief, 0.5 McFarland bacterial suspension in sterile physiologic saline solution was spread over Mueller-Hinton agar with an inoculating swab. The discs were placed on the media, and the plates were incubated for 16-18 hours on 37°C. A ≥ 5 mm difference in inhibition zone diameters around any of the antibiotic and the corresponding antibiotic + clavulanic acid discs was considered as a positive result for ESBL production.

Production of carbapenemases was presumed on the basis of the modified Hodge-test using 10 µg ertapenem discs (Bio-Rad, Hercules, CA, USA) [104]. Briefly, 0.5 McFarland *Escherichia coli* ATCC25922 suspension in sterile physiologic saline was spread over Mueller-Hinton agar. The ertapenem disc was placed in the middle of the plate and the test isolates were streaked on the plates outwards from the disc. The plates were incubated overnight on 37°C, and were checked for growth of *E. coli* near the ertapenem disc. Any overgrowth surrounding the test isolate was considered as a positive reaction and indicated the presence of a carbapenemase.

The results of the modified Hodge-test were confirmed with phenotypic inhibition assay (KPC+MBL Confirmation ID Kit, Rosco, Taastrup, Denmark). The assay was performed and interpreted according to the manufacturer's instructions.

The presence of various β-lactamase genes were investigated by PCR. Template DNA was prepared with boiling the pellets of 800 µl overnight bacterial culture resuspended in 800 µl sterile distilled water for 10 minutes. The PCR reactions were carried out in a final volume of 15 µl with primer concentration of 0.33 pmol/µl and 0.5 µl template DNA. Standard cycling conditions were used (predenaturation: 95°C 2 min, denaturation 95°C 30 sec, annealing T_a 30 sec, elongation 72°C 1 min 30 sec, final elongation 72°C 5 min; number of cycles: 35). The list of specific primers and the corresponding annealing temperatures (T_a) can be found in Table 8.

8. Table Primers used for β-lactamase detection

Primer	DNA sequence	T_a	Prod.	Ref.
CMY-1-F	5'-GCTGCTCAAGGAGCACAGGAT	52°C	520 bp	[105]
CMY-1-R	5'-CACATTGACATAGGTGTGGTGC			
CMY-2-F	5'-TGGCCAGAACTGACAGGCAA	52°C	462 bp	[105]
CMY-2-R	5'-TTTCTCCTGAACGTGGCTGGC			
CTX-M-1-F	5'-TTTGCGATGTGCAGTACCAGTAA	51°C	544 bp	[106]
CTX-M-1-R	5'-CGATATCGTTGGTGGTGCCATA			
CTX-M-2-F	5'-ATGTGCAGYACCAGTAARGTKATGGC	55°C	593 bp	[107]
CTX-M-2R	5'-TGGGTRAARTARGTSACCAGAAYCAGCGG			
CTX-M-G1-F	5'-ATGGTTAAAAAATCACTGCGYC	47°C	876 bp	[108]
CTX-M-G1-R	5'-TTACAAACCGTYGGTGACGATTT			
DHA-F	5'-AACTTTCACAGGTGTGCTGGGT	52°C	405 bp	[105]
DHA-R	5'-CCGTACGCATACTGGCTTTGC			
FOX-F	5'-AACATGGGGTATCAGGGAGATG	52°C	109 bp	[105]
FOX-R	5'-CAAAGCGCGTAACCGGATTGG			
IMP-F	5'-GGAATAGAGTGGCTTAAYT	52°C	232 bp	[109]
IMP-R	5'-TCGGTTTAAYAAAACAACCACC			
KPC-F	5'-CGTCTAGTTCTGCTGTCTTG	52°C	798 bp	[109]
KPC-R	5'-CTTGTCATCCTTGTTAGGCG			
NDM-F	5'-GGTTTGGCGATCTGGTTTTC	52°C	621 bp	[109]
NDM-R	5'-CGGAATGGCTCATCACGATC			
OXA-48-F	5'-GCGTGGTTAAGGATGAACAC	60°C	438 bp	[109]
OXA-48-R	5'-CATCAAGTTCAACCCAACCG			
SHV-1-F	5'-ATGCGTTATATTCGCCTGTG	49°C	865 bp	[110]
SHV-1-R	5'-GTTAGCGTTGCCAGTGCTCG			
SHV-2-F	5'-TTATCTCCCTGTTAGCCACC	49°C	796 bp	[111]
SHV-2-R	5'-GATTTGCTGATTTCGCTCGG			
TEM-1-F	5'-ATGAGTATTCAACATTTCCG	56°C	858 bp	[112]
TEM-1-R	5'-CCAATGCTTAATCAGTGAGG			
TEM-2-F	5'-GCGGAACCCCTATTTG	56°C	963 bp	[113]
TEM-2-R	5'-ACCATTGCTTAATCAGTGAG			
VIM-F	5'-GATGGTGTTTGGTCGCATA	52°C	390 bp	[109]
VIM-R	5'-CGAATGCGCAGCACCAG			

For study 1 (ESBL 2004-2008) primer pairs CTX-M-1, SHV-1 and TEM-1; for study 2 (CPKP 2009-2011) primer pairs CMY-1, CMY-2, CTX-M-2, DHA, FOX, IMP, KPC, NDM, OXA-48, SHV-2, TEM-2 and VIM were used.

Digestion of 7 µl of SHV PCR products with *Nhe*I for 30 min on 37°C was carried out in order to identify Gly238→Ser mutation of bla_{SHV} associated with the hydrolysis of third generation cephalosporins [114].

In study 1 (ESBL 2004-2008) bla_{CTX-M} and bla_{TEM} genes of major clones and bla_{SHV} genes of solely SHV-type ESBL producing minor clones were sequenced for single representatives using BigDye Terminator Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) and primer pairs SHV-1, TEM-1 and CTX-M-G1 respectively (Table 8).

In study 2 (CPKP 2009-2011) sequencing of the β-lactamase genes and the integron was performed for three and one selected isolates respectively at the National Center for Epidemiology (Budapest, Hungary) [99].

4.1.4 Antimicrobial susceptibility testing

For study 1 (ESBL 2004-2008) susceptibility to amikacin, ciprofloxacin, gentamicin, tobramycin, and trimethoprim/sulfamethoxazole was tested with disc diffusion method. In addition mimimum inhibitory concentration (MIC) of ciprofloxacin (Sigma-Aldrich, St. Louis, MO, USA) was determined by broth microdilution for 5 isolates of each major clone and every isolates of the minor clones.

For study 2 (CPKP 2009-2011) susceptibility to ertapenem, imipenem and meropenem was measured by MIC gradient test for all isolates. In addition, susceptibility to amikacin, chloramphenicol, colistin, fosfomycin and tigecycline was measured by MIC gradient test for blood and urine isolates, while susceptibility to gentamicin, tobramycin and ciprofloxacin was established by disc diffusion method.

Disc (Bio-Rad, Hercules, CA, USA) diffusion assays were performed with 0.5 McFarland standard inoculum on Mueller- Hinton agar media (Bio-Rad, Hercules, CA, USA) and incubation for 16-18 hours on 37°C. Broth microdilution was

carried out with 0.5 McFarland standard inoculum in Mueller- Hinton broth media (Oxoid, Altrincham, UK) and incubation for 16-18 hours on 37°C. For MIC gradient tests (Liofilchem, Roseto degli Abruzzi, Italy) instructions by the manufacturer were implemented. All results were interpreted according to EUCAST guidelines [115].

4.1.5 Virulence associated traits

For ESBL producing isolates the presence of several factors known or hypothesized to contribute to the virulence of *K. pneumoniae* was investigated. We focused on phenotypic detection in order to verify gene expression. In those cases were phenotypic tests were not available in our laboratory, we detected the relevant gene itself by PCR.

String-test was used to screen for hypermucoviscosity phenotype [116]. Fresh colonies on blood agar media were touched with a loop, and by lifting the loop upwards a “string” was tried to be pulled out. If the string was longer than 1 cm, the isolate was considered to be positive in the test.

Measurement of susceptibility to serum bactericidal activity was carried out with pooled human serum samples of healthy blood donors [117]. Density of overnight bacterial cultures were adjusted to match OD=0.4 at 600nm in phosphate buffered saline (PBS). This suspension was diluted to 100x in PBS, and then 25 µl of bacterial suspension was mixed with 75 µl of pooled human serum in a microtiter plate. The plates were incubated at 37°C for 180 minutes. Colony counting was performed at 0, 60 and 180 minutes. All isolates were tested in triplicates. The ratio of mean number of colony forming units at 60 and 180 minutes to mean number of colony forming units at 0 minute was evaluated for each isolate.

The production of enterobactin and aerobactin was established in a cross feeding bioassay [117]. For this test Luria agar supplemented with 275µM 2-2’-dipyridyl was prepared. Indicator strains (*Escherichia coli* H1939 for enterobactin and *E. coli* H1887 for aerobactin) were grown overnight, washed twice in sterile physiological saline solution, and their densities were adjusted to OD=0.1 at 600

nm. The bacterial suspensions were diluted 10x in physiological saline, and the dipyridyl containing media were overlaid with this suspension. The plates were left to dry on room temperature. The isolates to be investigated were prepared by washing 200 µl of overnight grown cultures twice in physiological salt solution, and after the final wash the pellets were resuspended in 1 ml. For each isolate 1 µl of the suspension was inoculated on the dipyridyl containing media overlaid with one of the indicator strains. The plates were incubated at 37°C, and were evaluated for growth of indicator strains around the place of inoculation after 24 and 48 hours. The assay was performed twice for each isolate.

Yeast cell co-agglutination in the presence or absence of 1% D-mannose was used to detect type 1 fimbria [118]. *Saccharomyces cervisiae* W303 was grown for 48 hours at 30°C. Cell density was adjusted to OD=1.1 at 600 nm in PBS, and the suspension was further concentrated with centrifugation and resuspension in 1/10 of the original volume. Bacteria were grown overnight on Brain-Heart Agar medium. Two loopful of a bacterial colony was picked up with a 1 µl standard loop and was suspended in 250 µl PBS. Then 40 µl bacterial suspension was mixed with 40 µl yeast suspension on a glass slide. The slides were evaluated for agglutination after 5 and 10 minutes of incubation. The experiment was repeated with 1% α-methyl-D-mannoside containing suspension in order to test for mannose sensitivity of the agglutination. The assay was performed three times for each isolate.

Expression of type 3 fimbria was evaluated by agglutination of tannic acid treated bovine erythrocytes [117]. For this test anticoagulated bovine blood was washed three times in PBS, and the pellet was finally suspended in 40x volume of PBS containing 0.003% tannic acid. The suspension was incubated for 75 minutes at 4°C. The bacteria were prepared as described for yeast cell co-agglutination. 50 µl of bacterial suspension was mixed with 50 µl tannic acid treated erythrocytes on glass slides. Agglutination was assessed after 5 and 10 minutes of incubation. All agglutination assays were carried out three times.

Microtiter plate assay was performed to estimate static biofilm forming capacity [119]. Assay plates were inoculated with 200 µl of overnight cultures diluted 1:100 in Luria Broth, and incubated at 37°C for 18 hours. The wells were

washed with PBS, fixed in 2% formalin in PBS for 2 minutes, and air dried. After staining with 0.13% crystal violet in 2% formalin and 0.5% etanol in PBS for 20 minutes, the wells war washed three times with PBS, and finally the bound dye was released from the biofilm by solubilisation of bacterial cells with 1% SDS and 50% ethanol in PBS for 2 hours. The OD of each well was measured at 595 nm with an ELISA plate reader. The mean OD_{595} of blank wells was extracted for every plate. Every isolate was tested in triplicate. The biofilm formation capacity was approximated by calculating the mean OD_{595} for each isolate.

The presence of the following virulence factors were detected by PCR: capsular serotype K1 (*magA*), capsular serotype K2 (*k2a*), regulator of mucoid phenotype (*rmpA*), yersiniabactin (*irp2-1*) and *Klebsiella* ferric iron uptake gene cluster (*kfuB*). Standard PCR conditions were used as written in section 3.4. The specific primers are listed in Table 9.

9. Table Primers used for detection of various virulence associated traits

Primer	DNA sequence	T_a	Prod.	Ref.
IRP-F	5'-ACCTCTTCACCCACCCTTCT	54°C	300 bp	[120]
IRP-R	5'-TTCAGGAAAATGGCAGGCGT			
K2A-F	5'-CAACCATGGTGGTCGATTAG	60°C	532 bp	[121]
K2A-R	5'-TGGTAGCCATATCCCTTTGG			
KFU-F	5'-GAAGTGACGCTGTTTCTGGC	60°C	797 bp	[37]
KFU-R	5'-TTTCGTGTGGCCAGTGACTC			
MAGA-F	5'-GGTGCTCTTTACATCATTGC	57°C	1281 bp	[121]
MAGA-R	5'-GCAATGGCCATTTGCGTTAG			
RMPA-F	5'-ACTGGGCTACCTCTGCTTCA	60°C	532 bp	[116]
RMPA-R	5'-CTTGCATGAGCCATCTTTCA			

4.1.6 Statistical methods

In order to test if the three major ESBL clones and the minor ESBL clones compounded were the same in the aspects of virulence associated factor content and occurrence of antibiotic resistance, probability values (p) were calculated with likelihood ratio test for categorical variables and Kruskal-Wallis-test for continuous variables (biofilm, serum resistance). All statistical computations were performed in SPSS 20. Statistical significance was established as $p<0.05$.

4.2 ESBL producing isolates (2004-2008)

4.2.1 Results

Based on macrorestriction profile analysis by PFGE the isolates were clustered into three major and eleven minor clones. The results of MLST and β-lactamase gene detection are presented in Table 10.

The major clones were identified and designated as Hungarian Epidemic Clone (HEC/ST15) for pulsotype PT-01, Epidemic Clone Pécs (ECP/ST101) for pulsotype PT-02 and Epidemic Clone II (EC II/ST147) for pulsotype PT-03.

The distribution of the different clones in time and place are summarised in Tables 11 & 12.

The results and statistical analysis of antimicrobial susceptibility testing and possession of virulence traits are presented in Tables 13 & 14.

10. Table Results of molecular typing and β-lactamase gene detection for ESBL producing *K. pneumoniae* isolates

	Pulsotype (PFGE)	Number of isolates	Result of MLST	β-lactamase genes detected
Major clones	PT-01	69	ST15	bla_{SHV} (*Nhe*I: negative), $bla_{CTX\text{-}M\text{-}15}$
	PT-02	10	ST101	bla_{SHV} (*Nhe*I: negative), $bla_{CTX\text{-}M\text{-}15}$ $bla_{TEM\text{-}1}$ (in six isolates)
	PT-03	9	ST147	bla_{SHV} (*Nhe*I: negative) $bla_{CTX\text{-}M}$
Minor clones	PT-04	2	-	bla_{SHV} (*Nhe*I: negative) $bla_{CTX\text{-}M}$
	PT-05	1	-	bla_{SHV} (*Nhe*I: negative) $bla_{CTX\text{-}M}$
	PT-06	1	-	bla_{SHV} (*Nhe*I: negative) $bla_{CTX\text{-}M}$
	PT-07	1	-	bla_{SHV} (*Nhe*I: negative) $bla_{CTX\text{-}M}$
	PT-08	1	-	bla_{SHV} (*Nhe*I: negative) $bla_{CTX\text{-}M}$
	PT-09	1	-	bla_{SHV} (*Nhe*I: negative) $bla_{CTX\text{-}M}$
	PT-10	2	ST1193	$bla_{SHV\text{-}5}$
	PT-11	1	ST34	$bla_{SHV\text{-}5}$
	PT-12	1	ST113	$bla_{SHV\text{-}5}$
	PT-13	1	ST323	$bla_{SHV\text{-}5}$
	PT-14	2	-	bla_{SHV} (*Nhe*I: positive) $bla_{CTX\text{-}M}$

11. Table Distribution of ESBL producing *K. pneumoniae* isolates among clones and year of isolation

Year	HEC ST15	ECP ST101	EC II ST147	minor clones
2004 (n=2)	0	1	0	1
2005 (n=9)	4	5	0	0
2006 (n=17)	12	3	0	2
2007 (n=47)	37	1	2	7
2008 (n=27)	16	0	7	4

12. Table Distribution of ESBL producing *K. pneumoniae* isolates among clones and departments

Department	HEC ST15	ECP ST101	EC II ST147	minor clones
Anaesthesia and Intensive Therapy (n=11)	6	0	0	5
Internal Medicine 1 (n=40)	27	9	1	3
Internal Medicine 2 (n=5)	3	1	1	0
Neurology (n=3)	2	0	0	1
Surgery (n=3)	3	0	0	0
Urology (n=35)	27	0	6	2
Other (n=5)	1	0	1	3

13. Table Susceptibility to various antimicrobial agents of ESBL producing *K. pneumoniae* clones

	HEC ST15 n=69	ECP ST101 n=10	EC II ST147 n=9	minor clones n=14	p
amikacin	51 (74%)	3 (30%)	8 (89%)	8 (57%)	0.018
gentamicin	11 (16%)	0 (0%)	1 (11%)	0 (0%)	0.070
tobramycin	4 (6%)	0 (0%)	0 (%)	0 (0%)	0.361
trimethoprim/ sulfamethoxazole	38 (55%)	1 (10%)	0 (0%)	9 (64%)	<0.001
ciprofloxacin	0 (0%)	0 (0%)	0 (0%)	9 (64%)	<0.001
ciprofloxacin MIC range	≥32 mg/L	≥32 mg/L	4-32 mg/L	0.06-32 mg/L	

14. Table Virulence associated factor content of ESBL producing *K. pneumoniae* clones

	HEC ST15 n=69	**ECP ST101 n=10**	**EC II ST147 n=9**	**minor clones n=14**	**p**
string-test	2 (3%)	0 (0%)	0 (0%)	2 (14%)	0.245
rmpA	0 (0%)	0 (0%)	0 (0%)	0 (0%)	-
magA	0 (0%)	0 (0%)	0 (0%)	0 (0%)	-
k2a	0 (0%)	0 (0%)	0 (0%)	0 (0%)	-
enterobactin	67 (97%)	5 (50%)	6 (67%)	14 (100%)	<0.001
aerobactin	1 (1%)	0 (0%)	0 (0%)	1 (7%)	0.566
kfuB	69 (100%)	10 (100%)	0 (0%)	3 (21%)	<0.001
irp2-1	0 (0%)	10 (100%)	0 (0%)	2 (14%)	<0.001
type 1 fimbria	67 (99%)*	10 (100%)	9 (100%)	13 (93%)	0.833
type 3 fimbria	65 (96%)*	4 (40%)	6 (67%)	6 (46%)	<0.001
biofilm (median)	3.526	2.112	2.463	1.262	<0.001
serum resistance at 60 min (median)	15.20%	5.40%	62.11%	1.46%	0.005
serum resistance at 180 min (median)	4.41%	4.09%	8.86%	0.09%	0.087

* n=68. One isolate showed autoaggregative properties.

4.2.2 Discussion

This study showed that the increase in the rate of ESBL producing isolates in the Clinical Centre University of Pécs between 2004 and 2008 was mostly explainable by the dissemination of CTX-M enzymes owed to the spread of three CTX-M-15 producing clones, namely HEC/ST15, ECP/ST101 and EC II/ST147, of which HEC/ST15 and EC II/ST147 were previously shown to be epidemic clones in Hungary [50]. Moreover CTX-M-15 producing ST15 can be regarded as an internationally disseminated, high risk, ESBL producing clone, as it was also detected in Italy, several Asian countries, Denmark, Portugal, Germany and Cuba [122–127]. Despite ST101 was shown to be a prevalent CTX-M-15 producer in several European countries (France, Italy and Greece), its presence in Hungary was first indicated by our investigations [122, 128, 129].

Beside the three major clones, several minor clones were identified. Among the minor clones also *bla*$_{\text{CTX-M}}$ was the most prevalent ESBL gene as more than half of them (6/11) were shown to be CTX-M producers. Only a smaller part (4 of 11) expressed *bla*$_{\text{SHV-5}}$, and just one clone harboured both ESBL-type *bla*$_{\text{SHV}}$ and *bla*$_{\text{CTX-M}}$.

Among SHV-5 producing minor clones a novel sequence type (ST1193) with allelic profile: 2-83-2-1-9-4-135 was found, and it harboured a unique variant of the *infB* allele (designated as number 83). The new sequence type and the *infB* allele were deposited and are publicly available at MLST database of Institut Pasteur (bigsdb.web.pasteur.fr).

Considering the spatial and timely distribution of the different clones in the Clinical Centre University of Pécs (Tables 11 and 12) it can be concluded that:

- ECP/ST101 might have been the dominant clone in 2004-2005 and was mainly related to Internal Medicine 1 and 2;
- HEC/ST15 started to prevail in all departments of the Clinical Centre during the initial period of this study, and continued to be the most prevalent ESBL producing clone of our institution;
- EC II/ST147 might have emerged around 2007 in our institution, and since then it has spread to several departments;

- minor clones were most common at the Department of Anaesthesia and Intensive Therapy and at other smaller departments.

Although we did not have the possibility to investigate every isolate originating from our institution, and for nine minor clone isolates the exact type of ESBL was not identified, local characteristics in the molecular epidemiology of ESBL producing *K. pneumoniae* isolates could be presumed when comparing our findings to national data. Two of the epidemic clones described (EC III/ST11 and EC IV/ST274) were not observed during our study period, and despite widespread dissemination of EC II/ST147 across the country in 2005, it was only detected first in 2007 in our institution possibly due to later importation or low incidence rates [50, 97]. According to this study HEC/ST15 and CTX-M-15 β-lactamases were the dominant clone and ESBL types, moreover SHV-5 was the only SHV-type ESBL identified in our institution, which corresponds to national data [50, 92].

While resistance to ciprofloxacin was universal in major clones, the majority (57.1%) of minor clone isolates showed wild-type phenotype according to epidemiological cut-off (ECOFF) values determined by EUCAST (www.eucast.org). Differences in the level of resistance to ciprofloxacin was suggested to be influenced by variations in fitness cost associated with the acquisition of fluoroquinolone resistance, and it was indicated that SHV-ESBL plasmids might be lost during the induction of high level resistance [130]. In our study high level ciprofloxacin resistance was not observed in SHV-5 producing isolates. The only resistant isolate showed low level resistance (MIC = 2 mg/L) and belonged to ST113.

Whereas resistance to gentamicin and tobramycin was similarly high in major and minor clones, rate of susceptibility to amikacin and trimethoprim/sulfamethoxazole varied across different clones. The majority of isolates belonging to major clones showed combined resistance to aminoglycosides and fluoroquinolones (ST15: 94%; ST101: 100%; ST147: 100%). Such a combination of resistance mechanism was seen less frequently in minor clone isolates (36%). The high rate of resistance to non-β-lactam agents might have contributed to the overusage of carbapenems.

Considering virulence associated trait content of the major clones, some general attributes could be seen: all three major clones showed high biofilm forming capacity and high rate of type 1 fimbria expression, on the other hand hypermucoviscosity phenotype, K1 or K2 serotype and aerobactin production was absent or rare. Beside these common features, several clonal characteristics could be recognised (Table 15).

15. Table Characteristic virulence associated factor content of ESBL producing *K. pneumoniae* clones (whereas symbols show frequency of: "+" = ≥90%; "+/-" = 90-50%; "-/+" = 49-10%; "-" ≤ 10% and "S" = median <50% for SR at 60 minutes and <5% for SR at 180 minutes, "R" = median ≥50% for SR at 60 minutes and ≥5% for SR at 180 minutes)

	enterobactin	**yersinibactin**	***kfu***	**type 3 fimbria**	**serum resistance**
HEC/ST15	+	-	+	+	S
ECP/ST101	+/-	+	+	-/+	S
EC II/ST147	+/-	-	-	+/-	I

The virulence associated traits identified in major clones were confirmed to play an important role during the pathogenesis of the following infections: type 1 fimbria in urinary tract infections, type 3 fimbria in catheter associated urinary tract infections, yersiniabactin in respiratory infections [24, 25, 33]. The possession of such virulence associated traits along with a multiresistant phenotype might render these ESBL producing major clones a successful nosocomial pathogen.

While some studies indicated that ESBL producing *K. pneumoniae* isolates (1) had higher rates of co-expression of type 1 and type 3 fimbria, (2) were more resistant to serum bactericidal activity or (3) showed increased adherence to and invasion of human epithelial cells, than non-ESBL producing ones [131–133], other studies suggested that different virulence factors might be associated with distinct clones or resistance plasmids [9, 134–136]. Our results also imply that the distribution of virulence associated traits might be diverse among different ESBL producing *K. pneumoniae* clones.

4.3 Carbapenemase producing isolates (2009-2011)

4.3.1 Results

According to macrorestriction profile analysis by PFGE, all but one isolate belonged to the formerly characterized HEC, which was confirmed with MLST to belong to ST15.

PCR showed the presence of CTX-M-, SHV-, TEM- and VIM-type β-lactamases for 100, 102, 102 and 101 isolates respectively. All isolates were negative for Gly238→Ser mutation of the *bla*$_{SHV}$ gene. The genes were identified as *bla*$_{CTX\text{-}M\text{-}15}$, *bla*$_{SHV\text{-}28}$, *bla*$_{TEM\text{-}1}$, and *bla*$_{VIM\text{-}4}$ according to sequencing.

The presence of a class 1 integron was revealed, which carried an *aac(6')-Ib* in the first gene cassette, followed by *bla*$_{VIM\text{-}4}$ in the second gene cassette. The integron was designated as In238b according to the Integrall database (integrall.bio.ua.pt).

The results of antibiotic susceptibility testing and phenotypic inhibition assay for VIM positive isolates are summarised in Tables 16-18. In the case of six isolates the results for amikacin were modified from susceptible to intermediate as stated in the EUCAST Expert Rule No. 12.7 [137].

16. Table Susceptibility to carbapenems of VIM producing *K. pneumoniae* isolates (n=101)

	Ertapenem	**Imipenem**	**Meropenem**
range (mg/L)	0.5-32	0.25-32	0.12-32
MIC_{50} (mg/L)	4	2	1
MIC_{90} (mg/L)	32	32	2
susceptible	7 (6.9%)	57 (56.4%)	91 (90.1%)
intermediate	10 (9.9%)	18 (17.8%)	8 (7.9%)
resistant	84 (83.2%)	26 (25.7%)	2 (2.0%)
above ECOFF	101 (100%)	61 (60.4%)	95 (94.1%)

17. Table Proportions of metallo-β-lactamase (MBL) positivity in the phenotypic inhibition assay and mean differences between inhibition zones of meropenem and meropenem + inhibitor in relation to meropenem MIC

Meropenem MIC (mg/L)	n	Boronic acid		Dipicolinic acid		Cloxacillin		MBL positive (%)
		mean (mm)	SD	mean (mm)	SD	mean (mm)	SD	
0.12	6	-0.8	1.169	2.2	0.983	0.0	0.693	0.0
0.25	21	0	1.284	2.8	1.209	0.6	1.284	14.3
0.5	21	0.4	1.284	4.6	1.028	1.1	0.944	52.4
1	30	0.6	1.382	4.8	1.315	1.5	1.042	63.3
2	13	0.4	0.870	4.9	1.256	1.2	1.235	69.2
4	8	0.3	1.753	4.9	0.991	1.0	1.512	75.0
32	2	0.5	0.707	6.0	1.414	0.0	0.0	100.0
all	101	0.3	1.300	4.2	1.567	1.0	1.177	49.0

18. Table Susceptibility to non-β-lactam antimicrobial agents of VIM producing *K. pneumoniae* isolates, n=82 (S = susceptible, I = intermediate, R = resistant)

	MIC range	MIC_{50}	MIC_{90}	S	I	R
ciprofloxacin	-	-	-	0 (0%)	0 (0%)	82 (100%)
gentamicin	-	-	-	6 (7.3%)	0 (0%)	76 (92.7%)
tobramycin	-	-	-	0 (0%)	0 (0%)	82 (100%)
amikacin	4-16 mg/L	8 mg/L	16 mg/L	66 (80.5%)	16 (19.5%)	0 (0%)
chloramphenicol	2-256 mg/L	8 mg/L	64 mg/L	42 (51.2%)	-	40 (48.8%)
colistin	0.5-4 mg/L	1 mg/L	1 mg/L	81 (98.8%)	-	1 (1.2%)
tigecycline	0.03-2 mg/L	0.5 mg/L	2 mg/L	66 (89.5%)	16 (19.5%)	0 (0%)
fosfomycin	4-256 mg/L	16 mg/L	256 mg/L	54 (65.9%)	-	28 (34.1%)

4.3.2 Discussion

This study showed that the emergence of carbapenemase-producing *K. pneumoniae* isolates in the Clinical Centre University of Pécs can be explained by the recent acquisition and expansion of *bla*$_{VIM-4}$ metallo-β-lactamase gene in the nationally disseminated and regionally dominant CTX-M-15 producing *K. pneumoniae* HEC/ST15. To our knowledge, this was the first description of VIM-4 production in ST15.

The *bla*$_{VIM-4}$ gene was located on a class 1 integron (In238b), wherewith identical ones were already identified in *Pseudomonas aeruginosa* (2002), *Aeromonas hydrophilia* (2005), *K. pneumoniae* ST11 (2009) and *Klebsiella oxytoca* (2009) in Hungary [99, 101, 138]. This indicates continuous circulation of In238b integron in our country.

The presence of In238b in the Clinical Centre University of Pécs was shown for *P. aeruginosa* in 2004 [139]. When this integron was introduced to the dominant ESBL producing *K. pneumoniae* clone of our institution, a remarkable expansion of VIM-4 production was observed. During the study period, of the 1654 patients from whom *K. pneumoniae* was isolated, 101 (6.1%) were confirmed by the present study to have VIM-producing isolates. The isolates with proven VIM-production originated from 12 distinct departments indicating a widespread dissemination within the Clinical Centre University of Pécs.

To date six different carbapenemase genes were identified in ST15, namely:

- NDM-1 in Canada, France, Morocco and Thailand [140–143];
- OXA-48 in Finland, France and Spain [144–146];
- OXA-162 in southern Hungary [100];
- VIM-1 in Spain [147];
- VIM-34 in Portugal [148] and
- VIM-4 described in the present study.

Considering this distribution two conclusions should be drawn. First, independent acquisition of different carbapenemase genes in the same sequence type indicates that ST15 has a great capacity to acquire different resistance plasmids, and can successively adapt to continuous antibiotic pressure. Second, VIM-4 production in ST15 seems to be confined to our region suggesting that it might have emerged locally with the attainment of In238b circulating in our country.

The genes *bla*$_{SHV-28}$ and *bla*$_{TEM-1}$ identified in ST15 in this study was noted earlier by others, and SHV-28 was proven to be non-ESBL type in another study [124, 149].

In our study the resistance conferred by the VIM metallo-β-lactamase was low level. For the majority of the isolates the MIC values of imipenem and meropenem were near the susceptible clinical breakpoint. The low level of resistance hindered detection by phenotypic inhibition assay as indicated by smaller difference between inhibition zones of meropenem and meropenem + dipicolinic acid at lower meropenem MIC (Table 17). This explains why a remarkable portion of isolates (51.0%) was not positive for metallo-β-lactamase production in the phenotypic inhibition assay, despite the production of the VIM enzyme could be demonstrated by the modified Hodge-test. Considering the low level of carbapenem resistance conferred by the VIM enzyme, the usage of meropenem ECOFF value proposed by EUCAST to screen for carbapenemase production, the usage of the modified Hodge-test and the simultaneous testing of susceptibility to the three carbapenem derivatives could be beneficial in the detection of VIM production [150].

The only VIM negative isolate was negative in the phenotypic inhibition test, was susceptible to all three carbapenem derivatives, and only the ertapenem MIC value was above ECOFF (www.eucast.org), therefore it can be presumed, that the modified Hodge-test gave a false positive result for this isolate.

For serious, life-threatening infections caused by carbapenemase producing *K. pneumoniae* isolates combination therapy should be given. A carbapenem based combination can be considered, if the isolate has a carbapenem MIC ≤4 mg/L [78]. This condition was met for 100/101 (99.0%) of the VIM positive

isolates, rendering these compounds to be a considerable choice in combination with other agents like colistin, tigecycline, fosfomycin, chloramphenicol, fluoroquinolones, and aminoglycosides.

In our study resistance to colistin was rare (1/82) and there were no isolates resistant to tigecycline. Despite their good in vitro activity both colistin and tigecycline have drawbacks. Colistin is nephrotoxic and neurotoxic, but recent advances in dosing regimens seem to abate this problem [151]. Tigecycline is approved for just three clinical syndromes (complicated intraabdominal infection, complicated skin and soft tissue infection and community acquired pneumonia) and most importantly lacks indications for treatment of sepsis, ventilator associated pneumonia or urinary tract infections [39].

Almost two third (65,9%) of the isolates in this study were susceptible to fosfomycin, making it a possible option for treatment of urinary tract infections, but it is only available as a per oral compound for short term treatment in our country, and per oral formulation is only proposed for treatment of uncomplicated urinary tract infections by EUCAST [115].

High portion of resistance (48.8%) and severe toxicity constrict the possible role of chloramphenicol in the treatment of infections caused by carbapenemase-producing *K. pneumoniae* in our institution.

All isolates were resistant to ciprofloxacin, which indicates resistance to all fluoroquinolone derivatives (EUCAST Expert Rule No. 13.5) [137]. Fluoroquinolone resistance in HEC/ST15 was shown to be due to mutations in *gyrA* and *parC* genes [50].

The possible usage of aminoglycosides is questioned by the presence of *aac(6')-Ib* in In238b. The AAC(6')-I enzyme is capable of modifying amikacin and tobramycin [137]. All isolates were resistant to tobramycin, but only 10 had amikacin MIC in the non-susceptible range. EUCAST Expert Rule No. 12.7 recommends the modification of amikacin results from susceptible to intermediate when the isolate is tobramycin resistant and gentamicin susceptible in order to indicate the possible modification of amikacin by an AAC(6')-I enzyme. This rule could be applied for six isolates, but for 66 isolates the possible

modification of amikacin was not indicated, because the phenotype described in the expert rule was possibly disguised by a gentamicin modifying enzyme.

Based on the results of molecular typing and antimicrobial susceptibility testing it can be affirmed that: if a severe infection by a carbapenemase producing *K. pneumonia*e isolate is suspected in our institution, an imipenem or meropenem plus colistin or tigecycline combination could be applicable as a first-line empiric therapy.

5. Conclusions

Our investigations were initiated to accumulate data on the molecular epidemiology of multiresistant *K. pneumoniae* isolates in the Clinical Centre University of Pécs. By observing the dissemination of isolates with acquired β-lactam resistance mechanisms within a single institution over a longer time period, a continuous progress in complexity and diversity, as well as certain regional characteristics, could be learned.

The persistent increase in the number of β-lactam resistant isolates in the Clinical Centre was partially attributed to the dissemination of CTX-M-15 producing epidemic clones (HEC/ST15, ECP/ST101 and EC II/ST147). This problem was further widened by the emergence and expansion of VIM-4 production in HEC/ST15 in 2009.

As the calculated incidence densities have shown, the infections and colonisations by these multiresistant clones happened in addition to the infections and colonisations by isolates without acquired β-lactam resistance mechanisms, and this way meant an extra burden on our patients. This point is particularly important when considering that these epidemic clones were shown in our studies to harbour a considerable range of virulence associated traits and resistance to non-β-lactam antimicrobial agents as well. Therefore any interventions aiming to hamper the dissemination of these multiresistant clones would be desirable.

Although we did not have the possibility to investigate every isolate from our institution, due to the selection of representative isolates the main tendencies and local features could be trailed.

As compared to nationwide studies the main regional characteristics pointed out by our studies were the presence of ECP/ST101 coupled with the absence of EC III/ST11 and EC IV/ST274, along with the high rate of carbapenemase production due to the endemism of $bla_{\text{VIM-4}}$ harbouring HEC/ST15.

Local variations were not only noted between regional and national data, but also within different departments of the Clinical Centre. While the ECP/ST101 seemed to be related to Internal Medicine, and the dissemination of EC II/ST147 was also constricted, on the other hand HEC/ST15 was generally widespread.

Sequence types identical to the ones detected in the present studies were also found to be prevalent ESBL and/or carbapenemase producers in various countries. The international dissemination of these high risk, multiresistant clones frames the questions whether (1) what determines the success and spread of particular clones, and (2) at what extent are the subpopulations within the same sequence type conserved or (3) how much do the regional variants of the same ST differ from each other. Profound understanding of this topic should possibly come from whole-genome sequencing and genomic epidemiology.

The local data we obtained should be valuable when amending infection control policies and empirical antimicrobial treatment recommendations to regional particularities. Nevertheless the real benefit of our studies are that we have obtained a comprehensive knowledge and a well-characterized strain collection of multidrug resistant *K. pneumoniae* isolates in our institution. This largely enlightens and accelerates the recognition and analysis of previously not seen or emerging resistance mechanisms in *K. pneumoniae*, as it has been done in the case of OXA-48 producing or colistin resistant isolates, so that appropriate infection control measures could be implicated as soon as possible (preliminary results were presented at the annual meeting of Hungarian Society for Microbiology in 2014 and at the scientific meeting of Hungarian Society of Clinical Microbiology and Infectious Diseases in 2015).

As *K. pneumoniae* shows a particular capability to continuously adapt to selective pressure meant by antibiotics, the local surveillance initiated by our studies is anticipated to be carried on and expanded in the future.

6. Novel findings

The main, original results of our investigations can be summarized as:

- first report on the presence of CTX-M-15 producing internationally disseminated ST101 high risk *K. pneumoniae* clone in Hungary;
- first detection of VIM-4 metallo-β-lactamase production in ST15 *K. pneumoniae* clone; and
- identification of novel *infB* allele (number: 83) and sequence type (ST1193, allelic profile: 2-83-2-1-9-4-135) in *K. pneumoniae*.

Additional novel results are:

- first comprehensive analysis on trends of β-lactam resistance rates of *K. pneumoniae* at the Clinical Centre University of Pécs
- first detailed description on dissemination of different multiresistant *K. pneumoniae* clones at the various departments of the Clinical Centre University of Pécs
- identification of diversities in virulence associated trait content of multiresistant *K. pneumoniae* clones at the Clinical Centre University of Pécs

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List of publications

Melegh S, Schneider G, Horváth M, Jakab F, Emődy L, Tigyi Z. “Identification and characterization of CTX-M-15 producing *Klebsiella pneumoniae* clone ST101 in a Hungarian university teaching hospital.” *Acta Microbiologica et Immunologica Hungarica* 2015 Sep; 62(3):pp233-245.

Melegh S, Kovács K, Gám T, Nyul A, Patkó B, Tóth A, Damjanova I, Mestyán G. “Emergence of VIM-4 metallo-β-lactamase-producing *Klebsiella pneumoniae* ST15 clone in the Clinical Centre University of Pécs, Hungary.” *Clinical Microbiology and Infection* 2014 Jan; 20(1):O27-9.

List of conference abstracts

Melegh S, Kovács K, Gám T, Nyul A, Patkó B, Tóth A, Damjanova I, Mestyán G. “Emergence of carbapenem resistant *Klebsiella pneumoniae* isolates in the Clinical Centre University of Pécs, Hungary.” *8th International Healthcare Infection Society Conference and Federation of Infection Societies Annual Conference,* November 2012, Liverpool, UK

Melegh S, Kovács K, Gám T, Nyul A, Patkó B, Damjanova I, Mestyán G. “A PTE klinikáin izolált karbapenem rezisztens *Klebsiella pneumoniae* törzsek karakterizálása: detektálási nehézségek és terápiás alternatívák.” *40th Congress of Hungarian Society of Clinical Microbiology and Infectious Diseases,* September 2012, Budapest, Hungary

Melegh S, Kovács K, Schneider G, Emődy L, Tigyi Z. “A *Klebsiella pneumoniae* törzsek rezisztenciájának változása a PTE klinikáin 2002-2010 között.” *39th Congress of Hungarian Society of Clinical Microbiology and Infectious Diseases,* September 2011, Pécs, Hungary

Melegh S, Schneider G, Veláncsics M, Damjanova I, Emődy L, Tigyi Z. "Dominancy of the N/ST15 pulsotype of extended spectrum β-lactamase producing *Klebsiella pneumoniae* isolates in the University Hospital of Pécs between 2004-2009." *16th International Congress of the Hungarian Society for Microbiology,* July 2011, Budapest, Hungary

Melegh S, Schneider G, Emődy L, Tigyi Z. "Evaluation of clonal diversity, biofilm formation and type 3 fimbria production by Extended Spectrum β-Lactamase positive *Klebsiella pneumoniae* isolates." *Annual Assembly of Hungarian Society for Microbiology,* October 2010, Keszthely, Hungary

Melegh S, Tigyi Z, Schneider G, Emődy L. "Biofilm formation and type 3 fimbria production by Extended Spectrum β-Lactamase producing *Klebsiella pneumoniae* strains." *Pathogenomics – From basic research to practical applications,* April 2010, Pécs, Hungary

Melegh S, Tigyi Z, Emődy L, Schneider G. "Characterisation of extended spectrum β-lactamase (ESBL) producing *Klebsiella pneumoniae* strains."
2nd Central European Forum for Microbiology, October 2009, Keszthely, Hungary

List of additional conference abstracts not included in this thesis

Melegh S, Nyul A, Kovács K, Ghidán Á, Jakab G, Dombrádi Z, Szabó J, Berta B, Lesinszki V, Pászti J, Tóth Á, Mestyán G. "A Tn1546 szerkezetének vizsgálata vancomycin rezisztens Enterococcus törzsekben" *43th Congress of Hungarian Society of Clinical Microbiology and Infectious Diseases,* September 2015, Nyíregyháza, Hungary

Melegh S, Nyul A, Kovács K, Gám T, Patkó B, Berta B, Lesinszki V, Pászti J, Tóth Á, Mestyán G. “Emergence of vancomycin resistant enterococci in the Clinical Centre University of Pécs, Hungary.” *25th European Congress of Clinical Microbiology and Infectious Diseases,* April 2015, Copenhagen, Denmark

Melegh S, Kovács K, Nyul A, Fenyvesi H, Jakab G, Szabó H, Jánvári L, Damjanova I, Tóth Á, Mestyán G. “Emergence of OXA-48-like carbapenemase producing *Klebsiella pneumoniae* in Pécs.” *Annual Assembly of Hungarian Society for Microbiology,* October 2014, Keszthely, Hungary

Kovács B, Rózsai B, Dorn Á, Kovács J, Kerényi M, **Melegh S**, Emődy L. “Investigations on virulence phenotypes in asymptomatic bacteriuria *Escherichia coli* isolates.” *Annual Assembly of Hungarian Society for Microbiology,* October 2012, Keszthely, Hungary

Kovács B, **Melegh S**, Rózsai B, Emődy L. “Aszimptomatikus bakteriuriát okozó *Escherichia coli* törzsek virulencia faktorainak vizsgálata.” *39th Congress of Hungarian Society of Clinical Microbiology and Infectious Diseases,* September 2011, Pécs, Hungary

Melegh S, Tigyi Z, Emődy L, Kocsis B, Schneider G. “Antibiotikum-oldatok 4^0C -on való eltarthatóságának vizsgálata mikrodilúciós módszerrel.” *39th Congress of Hungarian Society of Clinical Microbiology and Infectious Diseases,* September 2011, Pécs, Hungary

Tigyi Z, **Melegh S**, Pótó L, Emődy L. “Distribution of different iron uptake systems among *Klebsiella pneumoniae* strains isolated from wound, urinary and blood stream infections.” *16th International Congress of the Hungarian Society for Microbiology,* July 2011, Budapest, Hungary

References

[1] "European Centre for Disease Prevention and Control. Point prevalence survey of healthcare-associated infections and antimicrobial use in European acute care hospitals," Stockholm: ECDC, 2013.

[2] S. S. Magill, J. R. Edwards, W. Bamberg, Z. G. Beldavs, G. Dumyati, M. A. Kainer, R. Lynfield, M. Maloney, L. McAllister-Hollod, J. Nadle, S. M. Ray, D. L. Thompson, L. E. Wilson, and S. K. Fridkin, "Multistate point-prevalence survey of health care-associated infections.," *N. Engl. J. Med.*, vol. 370, no. 13, pp. 1198–208, Mar. 2014.

[3] R. Cantón, A. Novais, A. Valverde, E. Machado, L. Peixe, F. Baquero, and T. M. Coque, "Prevalence and spread of extended-spectrum beta-lactamase-producing Enterobacteriaceae in Europe.," *Clin. Microbiol. Infect.*, vol. 14 Suppl 1, pp. 144–53, Jan. 2008.

[4] R. Cantón, M. Akóva, Y. Carmeli, C. G. Giske, Y. Glupczynski, M. Gniadkowski, D. M. Livermore, V. Miriagou, T. Naas, G. M. Rossolini, Ø. Samuelsen, H. Seifert, N. Woodford, and P. Nordmann, "Rapid evolution and spread of carbapenemases among Enterobacteriaceae in Europe.," *Clin. Microbiol. Infect.*, vol. 18, no. 5, pp. 413–31, May 2012.

[5] V. James, Ed., *Manual of clinical microbiology*, 10th ed. Washington, DC: ASM Press, 2011.

[6] S. Brisse and J. Verhoef, "Phylogenetic diversity of *Klebsiella pneumoniae* and *Klebsiella oxytoca* clinical isolates revealed by randomly amplified polymorphic DNA, gyrA and parC genes sequencing and automated ribotyping.," *Int. J. Syst. Evol. Microbiol.*, vol. 51, no. Pt 3, pp. 915–24, May 2001.

[7] M. Rosenblueth, L. Martínez, J. Silva, and E. Martínez-Romero, "*Klebsiella variicola*, a novel species with clinical and plant-associated isolates.," *Syst. Appl. Microbiol.*, vol. 27, no. 1, pp. 27–35, Feb. 2004.

[8] S. Brisse, V. Passet, and P. A. D. Grimont, "Description of *Klebsiella quasipneumoniae* sp. nov., isolated from human infections, with two subspecies, *Klebsiella quasipneumoniae* subsp. *quasipneumoniae* subsp. nov. and *Klebsiella quasipneumoniae* subsp. *similipneumoniae* subsp. nov., and demonstration that *Klebsiella singaporensis* is a junior heterotypic synonym of *Klebsiella variicola*," *Int. J. Syst. Evol. Microbiol.*, vol. 64, no. Pt 9, pp. 3146–52, Sep. 2014.

[9] S. Brisse, C. Fevre, V. Passet, S. Issenhuth-Jeanjean, R. Tournebize, L. Diancourt, and P. Grimont, "Virulent clones of *Klebsiella pneumoniae*: identification and evolutionary scenario based on genomic and phenotypic characterization.," *PLoS One*, vol. 4, no. 3, p. e4982, Jan. 2009.

[10] M. S. Alves, R. C. da S. Dias, A. C. D. de Castro, L. W. Riley, and B. M. Moreira, “Identification of clinical isolates of indole-positive and indole-negative *Klebsiella* spp.,” *J. Clin. Microbiol.*, vol. 44, no. 10, pp. 3640–6, Oct. 2006.

[11] M. Maatallah, M. Vading, M. H. Kabir, A. Bakhrouf, M. Kalin, P. Nauclér, S. Brisse, and C. G. Giske, “*Klebsiella variicola* Is a Frequent Cause of Bloodstream Infection in the Stockholm Area, and Associated with Higher Mortality Compared to *K. pneumoniae*.,” *PLoS One*, vol. 9, no. 11, p. e113539, Jan. 2014.

[12] E. de Jong, A. S. de Jong, N. Smidts-van den Berg, and R. J. Rentenaar, “Differentiation of *Raoultella ornithinolytica/planticola* and *Klebsiella oxytoca* clinical isolates by matrix-assisted laser desorption/ionization-time of flight mass spectrometry.,” *Diagn. Microbiol. Infect. Dis.*, vol. 75, no. 4, pp. 431–3, Apr. 2013.

[13] S. Falkow, “Molecular Koch’s postulates applied to microbial pathogenicity.,” *Rev. Infect. Dis.*, vol. 10 Suppl 2, pp. S274–6, Jan. .

[14] P. Domenico, R. J. Salo, A. S. Cross, and B. A. Cunha, “Polysaccharide capsule-mediated resistance to opsonophagocytosis in *Klebsiella pneumoniae*.,” *Infect. Immun.*, vol. 62, no. 10, pp. 4495–9, Oct. 1994.

[15] R. Podschun and U. Ullmann, “*Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors.,” *Clin. Microbiol. Rev.*, vol. 11, no. 4, pp. 589–603, Oct. 1998.

[16] Y.-J. Pan, T.-L. Lin, Y.-H. Chen, C.-R. Hsu, P.-F. Hsieh, M.-C. Wu, and J.-T. Wang, “Capsular types of *Klebsiella pneumoniae* revisited by *wzc* sequencing.,” *PLoS One*, vol. 8, no. 12, p. e80670, Jan. 2013.

[17] K.-M. Yeh, A. Kurup, L. K. Siu, Y. L. Koh, C.-P. Fung, J.-C. Lin, T.-L. Chen, F.-Y. Chang, and T.-H. Koh, “Capsular serotype K1 or K2, rather than *magA* and *rmpA*, is a major virulence determinant for *Klebsiella pneumoniae* liver abscess in Singapore and Taiwan.,” *J. Clin. Microbiol.*, vol. 45, no. 2, pp. 466–71, Feb. 2007.

[18] K. Amako, Y. Meno, and A. Takade, “Fine structures of the capsules of *Klebsiella pneumoniae* and *Escherichia coli* K1.,” *J. Bacteriol.*, vol. 170, no. 10, pp. 4960–2, Oct. 1988.

[19] T. Kawai, “Hypermucoviscosity: an extremely sticky phenotype of *Klebsiella pneumoniae* associated with emerging destructive tissue abscess syndrome.,” *Clin. Infect. Dis.*, vol. 42, no. 10, pp. 1359–61, May 2006.

[20] D. S. Hansen, F. Mestre, S. Alberti, S. Hernández-Allés, D. Alvarez, A. Doménech-Sánchez, J. Gil, S. Merino, J. M. Tomás, and V. J. Benedí, "*Klebsiella pneumoniae* lipopolysaccharide O typing: revision of prototype strains and O-group distribution among clinical isolates from different sources and countries.," *J. Clin. Microbiol.*, vol. 37, no. 1, pp. 56–62, Jan. 1999.

[21] S. Merino, S. Camprubí, S. Albertí, V. J. Benedí, and J. M. Tomás, "Mechanisms of *Klebsiella pneumoniae* resistance to complement-mediated killing.," *Infect. Immun.*, vol. 60, no. 6, pp. 2529–35, Jun. 1992.

[22] J.-H. Chen, L. K. Siu, C.-P. Fung, J.-C. Lin, K.-M. Yeh, T.-L. Chen, Y.-K. Tsai, and F.-Y. Chang, "Contribution of outer membrane protein K36 to antimicrobial resistance and virulence in *Klebsiella pneumoniae*.," *J. Antimicrob. Chemother.*, vol. 65, no. 5, pp. 986–90, May 2010.

[23] C. Struve, M. Bojer, and K. A. Krogfelt, "Identification of a conserved chromosomal region encoding *Klebsiella pneumoniae* type 1 and type 3 fimbriae and assessment of the role of fimbriae in pathogenicity.," *Infect. Immun.*, vol. 77, no. 11, pp. 5016–24, Nov. 2009.

[24] C. Struve, M. Bojer, and K. A. Krogfelt, "Characterization of *Klebsiella pneumoniae* type 1 fimbriae by detection of phase variation during colonization and infection and impact on virulence.," *Infect. Immun.*, vol. 76, no. 9, pp. 4055–65, Sep. 2008.

[25] C. N. Murphy, M. S. Mortensen, K. A. Krogfelt, and S. Clegg, "Role of *Klebsiella pneumoniae* type 1 and type 3 fimbriae in colonizing silicone tubes implanted into the bladders of mice as a model of catheter-associated urinary tract infections.," *Infect. Immun.*, vol. 81, no. 8, pp. 3009–17, Aug. 2013.

[26] R. M. Donlan, "Biofilms: microbial life on surfaces.," *Emerg. Infect. Dis.*, vol. 8, no. 9, pp. 881–90, Sep. 2002.

[27] M. Abdallah, C. Benoliel, D. Drider, P. Dhulster, and N.-E. Chihib, "Biofilm formation and persistence on abiotic surfaces in the context of food and medical environments.," *Arch. Microbiol.*, vol. 196, no. 7, pp. 453–72, Jul. 2014.

[28] M. Bandeira, P. A. Carvalho, A. Duarte, and L. Jordao, "Exploring Dangerous Connections between *Klebsiella pneumoniae* Biofilms and Healthcare-Associated Infections.," *Pathog. (Basel, Switzerland)*, vol. 3, no. 3, pp. 720–31, Jan. 2014.

[29] M.-C. Wu, Y.-C. Chen, T.-L. Lin, P.-F. Hsieh, and J.-T. Wang, "Cellobiose-specific phosphotransferase system of *Klebsiella pneumoniae* and its importance in biofilm formation and virulence.," *Infect. Immun.*, vol. 80, no. 7, pp. 2464–72, Jul. 2012.

[30] Q. Kong, J. M. Beanan, R. Olson, U. Macdonald, A. S. Shon, D. J. Metzger, A. O. Pomakov, and T. A. Russo, “Biofilm formed by a hypervirulent (hypermucoviscous) variant of *Klebsiella pneumoniae* does not enhance serum resistance or survival in an in vivo abscess model.,” *Virulence*, vol. 3, no. 3, pp. 309–18, May 2012.

[31] S. G. Stahlhut, C. Schroll, M. Harmsen, C. Struve, and K. A. Krogfelt, “Screening for genes involved in *Klebsiella pneumoniae* biofilm formation using a fosmid library.,” *FEMS Immunol. Med. Microbiol.*, vol. 59, no. 3, pp. 521–4, Aug. 2010.

[32] J. D. Boddicker, R. A. Anderson, J. Jagnow, and S. Clegg, “Signature-tagged mutagenesis of *Klebsiella pneumoniae* to identify genes that influence biofilm formation on extracellular matrix material.,” *Infect. Immun.*, vol. 74, no. 8, pp. 4590–7, Aug. 2006.

[33] M. A. Bachman, S. Lenio, L. Schmidt, J. E. Oyler, and J. N. Weiser, “Interaction of lipocalin 2, transferrin, and siderophores determines the replicative niche of *Klebsiella pneumoniae* during pneumonia.,” *MBio*, vol. 3, no. 6, Jan. 2012.

[34] M. A. Bachman, J. E. Oyler, S. H. Burns, M. Caza, F. Lépine, C. M. Dozois, and J. N. Weiser, “*Klebsiella pneumoniae* yersiniabactin promotes respiratory tract infection through evasion of lipocalin 2.,” *Infect. Immun.*, vol. 79, no. 8, pp. 3309–16, Aug. 2011.

[35] M. A. Fischbach, H. Lin, L. Zhou, Y. Yu, R. J. Abergel, D. R. Liu, K. N. Raymond, B. L. Wanner, R. K. Strong, C. T. Walsh, A. Aderem, and K. D. Smith, “The pathogen-associated *iroA* gene cluster mediates bacterial evasion of lipocalin 2.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 103, no. 44, pp. 16502–7, Oct. 2006.

[36] T. A. Russo, R. Olson, U. Macdonald, D. Metzger, L. M. Maltese, E. J. Drake, and A. M. Gulick, “Aerobactin mediates virulence and accounts for increased siderophore production under iron-limiting conditions by hypervirulent (hypermucoviscous) *Klebsiella pneumoniae*.,” *Infect. Immun.*, vol. 82, no. 6, pp. 2356–67, Jun. 2014.

[37] L.-C. Ma, C.-T. Fang, C.-Z. Lee, C.-T. Shun, and J.-T. Wang, “Genomic heterogeneity in *Klebsiella pneumoniae* strains is associated with primary pyogenic liver abscess and metastatic infection.,” *J. Infect. Dis.*, vol. 192, no. 1, pp. 117–28, Jul. 2005.

[38] H.-L. Tang, M.-K. Chiang, W.-J. Liou, Y.-T. Chen, H.-L. Peng, C.-S. Chiou, K.-S. Liu, M.-C. Lu, K.-C. Tung, and Y.-C. Lai, “Correlation between *Klebsiella pneumoniae* carrying pLVPK-derived loci and abscess formation.,” *Eur. J. Clin. Microbiol. Infect. Dis.*, vol. 29, no. 6, pp. 689–98, Jun. 2010.

[39] *Mandell, Douglas and Bennett’s Principle and Practice of Infectious Diseases*, 8th ed. Philadelphia, PA: Saunders, 2015.

[40] W. R. Jarvis, V. P. Munn, A. K. Highsmith, D. H. Culver, and J. M. Hughes, “The epidemiology of nosocomial infections caused by *Klebsiella pneumoniae*.,” *Infect. Control*, vol. 6, no. 2, pp. 68–74, Feb. 1985.

[41] L. K. Siu, K.-M. Yeh, J.-C. Lin, C.-P. Fung, and F.-Y. Chang, “*Klebsiella pneumoniae* liver abscess: a new invasive syndrome.,” *Lancet. Infect. Dis.*, vol. 12, no. 11, pp. 881–7, Nov. 2012.

[42] European Centre for Disease Prevention and Control, *Surveillance of antimicrobial consumption in Europe 2011*. Stockholm: ECDC, 2014.

[43] R. P. Ambler, “The structure of beta-lactamases.,” *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, vol. 289, no. 1036, pp. 321–31, May 1980.

[44] K. Bush and G. A. Jacoby, “Updated functional classification of beta-lactamases.,” *Antimicrob. Agents Chemother.*, vol. 54, no. 3, pp. 969–76, Mar. 2010.

[45] S. Haeggman, S. Löfdahl, A. Paauw, J. Verhoef, and S. Brisse, “Diversity and evolution of the class A chromosomal beta-lactamase gene in *Klebsiella pneumoniae*.,” *Antimicrob. Agents Chemother.*, vol. 48, no. 7, pp. 2400–8, Jul. 2004.

[46] “National Nosocomial Surveillance System. National Center for Epidemiology, Budapest, Hungary.” [Online]. Available: www.oek.hu.

[47] D. M. Livermore, “Defining an extended-spectrum beta-lactamase.,” *Clin. Microbiol. Infect.*, vol. 14 Suppl 1, pp. 3–10, Jan. 2008.

[48] L. Drieux, F. Brossier, W. Sougakoff, and V. Jarlier, “Phenotypic detection of extended-spectrum beta-lactamase production in Enterobacteriaceae: review and bench guide.,” *Clin. Microbiol. Infect.*, vol. 14 Suppl 1, pp. 90–103, Jan. 2008.

[49] M. G. P. Page, “Extended-spectrum beta-lactamases: structure and kinetic mechanism.,” *Clin. Microbiol. Infect.*, vol. 14 Suppl 1, pp. 63–74, Jan. 2008.

[50] I. Damjanova, A. Tóth, J. Pászti, G. Hajbel-Vékony, M. Jakab, J. Berta, H. Milch, and M. Füzi, “Expansion and countrywide dissemination of ST11, ST15 and ST147 ciprofloxacin-resistant CTX-M-15-type beta-lactamase-producing *Klebsiella pneumoniae* epidemic clones in Hungary in 2005--the new ‘MRSAs’?,” *J. Antimicrob. Chemother.*, vol. 62, no. 5, pp. 978–85, Nov. 2008.

[51] G. A. Jacoby, “AmpC beta-lactamases.,” *Clin. Microbiol. Rev.*, vol. 22, no. 1, pp. 161–82, Jan. 2009.

[52] C. Mata, E. Miró, A. Rivera, B. Mirelis, P. Coll, and F. Navarro, “Prevalence of acquired AmpC beta-lactamases in Enterobacteriaceae lacking inducible chromosomal *ampC* genes at a Spanish hospital from 1999 to 2007.,” *Clin. Microbiol. Infect.*, vol. 16, no. 5, pp. 472–6, May 2010.

[53] N. Woodford, J. W. T. Dallow, R. L. R. Hill, M.-F. I. Palepou, R. Pike, M. E. Ward, M. Warner, and D. M. Livermore, “Ertapenem resistance among *Klebsiella* and *Enterobacter* submitted in the UK to a reference laboratory.,” *Int. J. Antimicrob. Agents*, vol. 29, no. 4, pp. 456–9, Apr. 2007.

[54] Z. Pragai and E. Nagy, “In-vitro selection of porin-deficient mutants of two strains of *Klebsiella pneumoniae* with reduced susceptibilities to meropenem, but not to imipenem.,” *J. Antimicrob. Chemother.*, vol. 42, no. 6, pp. 821–4, Dec. 1998.

[55] S. Hernández-Allés, S. Albertí, D. Alvarez, A. Doménech-Sánchez, L. Martínez-Martínez, J. Gil, J. M. Tomás, and V. J. Benedí, “Porin expression in clinical isolates of *Klebsiella pneumoniae*.,” *Microbiology*, vol. 145 (Pt 3, pp. 673–9, Mar. 1999.

[56] L. García-Sureda, A. Doménech-Sánchez, M. Barbier, C. Juan, J. Gascó, and S. Albertí, “OmpK26, a novel porin associated with carbapenem resistance in *Klebsiella pneumoniae*.,” *Antimicrob. Agents Chemother.*, vol. 55, no. 10, pp. 4742–7, Oct. 2011.

[57] G. Cornaglia, H. Giamarellou, and G. M. Rossolini, “Metallo-β-lactamases: a last frontier for β-lactams?,” *Lancet. Infect. Dis.*, vol. 11, no. 5, pp. 381–93, May 2011.

[58] P. Nordmann, M. Gniadkowski, C. G. Giske, L. Poirel, N. Woodford, and V. Miriagou, “Identification and screening of carbapenemase-producing Enterobacteriaceae.,” *Clin. Microbiol. Infect.*, vol. 18, no. 5, pp. 432–8, May 2012.

[59] G. A. Jacoby, “Mechanisms of resistance to quinolones.,” *Clin. Infect. Dis.*, vol. 41 Suppl 2, pp. S120–6, Jul. 2005.

[60] H. Y. Yang, Y. S. Nam, and H. J. Lee, “Prevalence of plasmid-mediated quinolone resistance genes among ciprofloxacin-nonsusceptible *Escherichia coli* and *Klebsiella pneumoniae* isolated from blood cultures in Korea.,” *Can. J. Infect. Dis. Med. Microbiol.*, vol. 25, no. 3, pp. 163–9, May 2014.

[61] J. M. Rodríguez-Martínez, M. E. Cano, C. Velasco, L. Martínez-Martínez, and A. Pascual, “Plasmid-mediated quinolone resistance: an update.,” *J. Infect. Chemother.*, vol. 17, no. 2, pp. 149–82, Apr. 2011.

[62] K. H. Mayer, “Review of epidemic aminoglycoside resistance worldwide.,” *Am. J. Med.*, vol. 80, no. 6B, pp. 56–64, Jun. 1986.

[63] E. Machado, T. M. Coque, R. Cantón, F. Baquero, J. C. Sousa, and L. Peixe, “Dissemination in Portugal of CTX-M-15-, OXA-1-, and TEM-1-producing Enterobacteriaceae strains containing the *aac(6’)-Ib-cr* gene, which encodes an aminoglycoside- and fluoroquinolone-modifying enzyme.,” *Antimicrob. Agents Chemother.*, vol. 50, no. 9, pp. 3220–1, Sep. 2006.

[64] Y. Zhou, H. Yu, Q. Guo, X. Xu, X. Ye, S. Wu, Y. Guo, and M. Wang, “Distribution of 16S rRNA methylases among different species of Gram-negative bacilli with high-level resistance to aminoglycosides.,” *Eur. J. Clin. Microbiol. Infect. Dis.*, vol. 29, no. 11, pp. 1349–53, Nov. 2010.

[65] S. Schwarz, C. Kehrenberg, B. Doublet, and A. Cloeckaert, “Molecular basis of bacterial resistance to chloramphenicol and florfenicol.,” *FEMS Microbiol. Rev.*, vol. 28, no. 5, pp. 519–42, Nov. 2004.

[66] Y.-M. Ah, A.-J. Kim, and J.-Y. Lee, “Colistin resistance in *Klebsiella pneumoniae*.,” *Int. J. Antimicrob. Agents*, vol. 44, no. 1, pp. 8–15, Jul. 2014.

[67] M. S. Wright, Y. Suzuki, M. B. Jones, S. H. Marshall, S. D. Rudin, D. van Duin, K. Kaye, M. R. Jacobs, R. A. Bonomo, and M. D. Adams, “Genomic and transcriptomic analyses of colistin-resistant clinical isolates of *Klebsiella pneumoniae* reveal multiple pathways of resistance.,” *Antimicrob. Agents Chemother.*, Nov. 2014.

[68] D. E. Karageorgopoulos, R. Wang, X.-H. Yu, and M. E. Falagas, “Fosfomycin: evaluation of the published evidence on the emergence of antimicrobial resistance in Gram-negative pathogens.,” *J. Antimicrob. Chemother.*, vol. 67, no. 2, pp. 255–68, Feb. 2012.

[69] R. L. Then, “Mechanisms of resistance to trimethoprim, the sulfonamides, and trimethoprim-sulfamethoxazole.,” *Rev. Infect. Dis.*, vol. 4, no. 2, pp. 261–9, Jan. .

[70] M. C. Roberts, “Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution.,” *FEMS Microbiol. Rev.*, vol. 19, no. 1, pp. 1–24, Oct. 1996.

[71] X. Wang, H. Chen, Y. Zhang, Q. Wang, C. Zhao, H. Li, W. He, F. Zhang, Z. Wang, S. Li, and H. Wang, “Genetic characterisation of clinical *Klebsiella pneumoniae* isolates with reduced susceptibility to tigecycline: Role of the global regulator RamA and its local repressor RamR.,” *Int. J. Antimicrob. Agents*, Jan. 2015.

[72] K. Z. Vardakas, G. S. Tansarli, P. I. Rafailidis, and M. E. Falagas, “Carbapenems versus alternative antibiotics for the treatment of bacteraemia due to Enterobacteriaceae producing extended-spectrum β-lactamases: a systematic review and meta-analysis.,” *J. Antimicrob. Chemother.*, vol. 67, no. 12, pp. 2793–803, Dec. 2012.

[73] K. Yang and B. J. Guglielmo, “Diagnosis and treatment of extended-spectrum and AmpC beta-lactamase-producing organisms.,” *Ann. Pharmacother.*, vol. 41, no. 9, pp. 1427–35, Sep. 2007.

[74] J. Garau, “Other antimicrobials of interest in the era of extended-spectrum beta-lactamases: fosfomycin, nitrofurantoin and tigecycline.,” *Clin. Microbiol. Infect.*, vol. 14 Suppl 1, pp. 198–202, Jan. 2008.

[75] D. M. Livermore, R. Hope, S. Mushtaq, and M. Warner, “Orthodox and unorthodox clavulanate combinations against extended-spectrum beta-lactamase producers.,” *Clin. Microbiol. Infect.*, vol. 14 Suppl 1, pp. 189–93, Jan. 2008.

[76] L. R. Peterson, “Antibiotic policy and prescribing strategies for therapy of extended-spectrum beta-lactamase-producing Enterobacteriaceae: the role of piperacillin-tazobactam.,” *Clin. Microbiol. Infect.*, vol. 14 Suppl 1, pp. 181–4, Jan. 2008.

[77] M. E. Falagas, P. Lourida, P. Poulikakos, P. I. Rafailidis, and G. S. Tansarli, “Antibiotic treatment of infections due to carbapenem-resistant Enterobacteriaceae: systematic evaluation of the available evidence.,” *Antimicrob. Agents Chemother.*, vol. 58, no. 2, pp. 654–63, Jan. 2014.

[78] G. L. Daikos, S. Tsaousi, L. S. Tzouvelekis, I. Anyfantis, M. Psichogiou, A. Argyropoulou, I. Stefanou, V. Sypsa, V. Miriagou, M. Nepka, S. Georgiadou, A. Markogiannakis, D. Goukos, and A. Skoutelis, “Carbapenemase-producing *Klebsiella pneumoniae* bloodstream infections: lowering mortality by antibiotic combination schemes and the role of carbapenems.,” *Antimicrob. Agents Chemother.*, vol. 58, no. 4, pp. 2322–8, Jan. 2014.

[79] M. Tumbarello, P. Viale, C. Viscoli, E. M. Trecarichi, F. Tumietto, A. Marchese, T. Spanu, S. Ambretti, F. Ginocchio, F. Cristini, A. R. Losito, S. Tedeschi, R. Cauda, and M. Bassetti, “Predictors of mortality in bloodstream infections caused by *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae*: importance of combination therapy.,” *Clin. Infect. Dis.*, vol. 55, no. 7, pp. 943–50, Oct. 2012.

[80] E. Szilágyi, M. Füzi, K. Böröcz, A. Kurcz, A. Tóth, and K. Nagy, “Risk factors and outcomes for bloodstream infections with extended-spectrum beta -lactamase-producing *Klebsiella pneumoniae* ; Findings of the nosocomial surveillance system in Hungary.,” *Acta Microbiol. Immunol. Hung.*, vol. 56, no. 3, pp. 251–62, Sep. 2009.

[81] R. E. Warren, G. Harvey, R. Carr, D. Ward, and A. Doroshenko, “Control of infections due to extended-spectrum beta-lactamase-producing organisms in hospitals and the community.,” *Clin. Microbiol. Infect.*, vol. 14 Suppl 1, pp. 124–33, Jan. 2008.

[82] M. Akova, G. L. Daikos, L. Tzouvelekis, and Y. Carmeli, “Interventional strategies and current clinical experience with carbapenemase-producing Gram-negative bacteria.,” *Clin. Microbiol. Infect.*, vol. 18, no. 5, pp. 439–48, May 2012.

[83] B. Foxman and L. Riley, “Molecular epidemiology: focus on infection.,” *Am. J. Epidemiol.*, vol. 153, no. 12, pp. 1135–41, Jun. 2001.

[84] A. van Belkum, P. T. Tassios, L. Dijkshoorn, S. Haeggman, B. Cookson, N. K. Fry, V. Fussing, J. Green, E. Feil, P. Gerner-Smidt, S. Brisse, and M. Struelens, “Guidelines for the validation and application of typing methods for use in bacterial epidemiology.,” *Clin. Microbiol. Infect.*, vol. 13 Suppl 3, pp. 1–46, Oct. 2007.

[85] M. J. Struelens and S. Brisse, “From molecular to genomic epidemiology: transforming surveillance and control of infectious diseases.,” *Euro Surveill.*, vol. 18, no. 4, p. 20386, Jan. 2013.

[86] “Standard Operating Procedure for PulseNet PFGE of *Escherichia coli* O157:H7, *Escherichia coli* non-O157 (STEC), *Salmonella* serotypes, *Shigella sonnei* and *Shigella flexneri*,” 2013. [Online]. Available: www.cdc.gov/pulsenet. [Accessed: 22-Jun-2015].

[87] L. Diancourt, V. Passet, J. Verhoef, P. A. D. Grimont, and S. Brisse, “Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates.,” *J. Clin. Microbiol.*, vol. 43, no. 8, pp. 4178–82, Aug. 2005.

[88] Z. Gál, D. Szabó, P. Kovács, F. Hernádi, B. Tóth-Martinez, and F. Rozgonyi, “Beta-Lactam susceptibility patterns and investigation of cephalosporin hydrolysing beta-lactamases of Gram-negative extraintestinal clinical isolates.,” *Int. J. Antimicrob. Agents*, vol. 16, no. 4, pp. 395–400, Dec. 2000.

[89] Z. Pragai, Z. Koczian, and E. Nagy, “Characterization of the extended-spectrum beta-lactamases and determination of the antibiotic susceptibilities of *Klebsiella pneumoniae* isolates in Hungary.,” *J. Antimicrob. Chemother.*, vol. 42, no. 3, pp. 401–3, Sep. 1998.

[90] D. Szabó, Z. Filetóth, J. Szentandrássy, M. Némedi, E. Tóth, C. Jeney, G. Kispál, and F. Rozgonyi, “Molecular epidemiology of a cluster of cases due to *Klebsiella pneumoniae* producing SHV-5 extended-spectrum beta-lactamase in the premature intensive care unit of a Hungarian hospital.,” *J. Clin. Microbiol.*, vol. 37, no. 12, pp. 4167–9, Dec. 1999.

[91] K. Kristóf, D. Szabó, J. W. Marsh, V. Cser, L. Janik, F. Rozgonyi, A. Nobilis, K. Nagy, and D. L. Paterson, “Extended-spectrum beta-lactamase-producing *Klebsiella* spp. in a neonatal intensive care unit: risk factors for the infection and the dynamics of the molecular epidemiology.,” *Eur. J. Clin. Microbiol. Infect. Dis.*, vol. 26, no. 8, pp. 563–70, Aug. 2007.

[92] A. Tóth, M. Gacs, K. Márialigeti, G. Cech, and M. Füzi, “Occurrence and regional distribution of SHV-type extended-spectrum beta-lactamases in Hungary.,” *Eur. J. Clin. Microbiol. Infect. Dis.*, vol. 24, no. 4, pp. 284–7, Apr. 2005.

[93] I. Damjanova, A. Tóth, J. Pászti, M. Jakab, H. Milch, A. Bauernfeind, and M. Füzi, “Epidemiology of SHV-type beta-lactamase-producing *Klebsiella* spp. from outbreaks in five geographically distant Hungarian neonatal intensive care units: widespread dissemination of epidemic R-plasmids.,” *Int. J. Antimicrob. Agents*, vol. 29, no. 6, pp. 665–71, Jun. 2007.

[94] I. Damjanova, A. Tóth, J. Pászti, A. Bauernfeind, and M. Füzi, “Nationwide spread of clonally related CTX-M-15-producing multidrug-resistant *Klebsiella pneumoniae* strains in Hungary.,” *Eur. J. Clin. Microbiol. Infect. Dis.*, vol. 25, no. 4, pp. 275–8, Apr. 2006.

[95] E. Szilágyi, M. Füzi, I. Damjanova, K. Böröcz, K. Szonyi, A. Tóth, and K. Nagy, “Investigation of extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* outbreaks in Hungary between 2005 and 2008.,” *Acta Microbiol. Immunol. Hung.*, vol. 57, no. 1, pp. 43–53, Mar. 2010.

[96] D. Szabó, B. Kocsis, L. Rókusz, J. Szentandrássy, K. Katona, K. Kristóf, and K. Nagy, “First detection of plasmid-mediated, quinolone resistance determinants *qnrA*, *qnrB*, *qnrS* and *aac(6’)-Ib-cr* in extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae in Budapest, Hungary.,” *J. Antimicrob. Chemother.*, vol. 62, no. 3, pp. 630–2, Sep. 2008.

[97] I. Damjanova, A. Tóth, E. Kenesei, M. Köhalmi, P. Szántai, M. Füzi, and J. Pászti, “Dissemination of ST274 *Klebsiella pneumoniae* epidemic clone in newborn and adult hospital settings harbouring SHV-2A or CTX-M-15 type extended spectrum β-lactamases-producing known plasmids.,” *Eur. J. Microbiol. Immunol. (Bp).*, vol. 1, no. 3, pp. 223–7, Sep. 2011.

[98] A. Tóth, I. Damjanova, E. Puskás, L. Jánvári, M. Farkas, A. Dobák, K. Böröcz, and J. Pászti, “Emergence of a colistin-resistant KPC-2-producing *Klebsiella pneumoniae* ST258 clone in Hungary.,” *Eur. J. Clin. Microbiol. Infect. Dis.*, vol. 29, no. 7, pp. 765–9, Jul. 2010.

[99] K. Kristóf, A. Tóth, I. Damjanova, L. Jánvári, M. Konkoly-Thege, B. Kocsis, R. Koncan, G. Cornaglia, E. Szego, K. Nagy, and D. Szabó, “Identification of a *bla*VIM-4 gene in the internationally successful *Klebsiella pneumoniae* ST11 clone and in a *Klebsiella oxytoca* strain in Hungary.,” *J. Antimicrob. Chemother.*, vol. 65, no. 6, pp. 1303–5, Jun. 2010.

[100] L. Jánvári, I. Damjanova, A. Lázár, K. Rácz, B. Kocsis, E. Urbán, and Á. Tóth, “Emergence of OXA-162-producing *Klebsiella pneumoniae* in Hungary.,” *Scand. J. Infect. Dis.*, vol. 46, no. 4, pp. 320–4, Apr. 2014.

[101] B. Libisch, M. Gacs, K. Csiszár, M. Muzslay, L. Rókusz, and M. Füzi, “Isolation of an integron-borne *bla*VIM-4 type metallo-beta-lactamase gene from a carbapenem-resistant *Pseudomonas aeruginosa* clinical isolate in Hungary.,” *Antimicrob. Agents Chemother.*, vol. 48, no. 9, pp. 3576–8, Sep. 2004.

[102] “National Bacteriological Surveillance Management Team. NBS Annual reports. National Center for Epidemiology, Budapest, Hungary.” [Online]. Available: www.oek.hu. [Accessed: 22-Jun-2015].

[103] G. Cornaglia, W. Hryniewicz, V. Jarlier, G. Kahlmeter, H. Mittermayer, L. Stratchounski, and F. Baquero, “European recommendations for antimicrobial resistance surveillance.,” *Clin. Microbiol. Infect.*, vol. 10, no. 4, pp. 349–83, Apr. 2004.

[104] *CLSI. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-first Informational Supplement. Document M100-S21.* Wayne, PA: CLSI, 2009.

[105] F. J. Pérez-Pérez and N. D. Hanson, “Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR.,” *J. Clin. Microbiol.*, vol. 40, no. 6, pp. 2153–62, Jun. 2002.

[106] M. Edelstein, M. Pimkin, I. Palagin, I. Edelstein, and L. Stratchounski, “Prevalence and molecular epidemiology of CTX-M extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in Russian hospitals.,” *Antimicrob. Agents Chemother.*, vol. 47, no. 12, pp. 3724–32, Dec. 2003.

[107] H. Hasman, D. Mevius, K. Veldman, I. Olesen, and F. M. Aarestrup, “Beta-Lactamases among extended-spectrum beta-lactamase (ESBL)-resistant *Salmonella* from poultry, poultry products and human patients in The Netherlands.,” *J. Antimicrob. Chemother.*, vol. 56, no. 1, pp. 115–21, Jul. 2005.

[108] R. L. Jørgensen, J. B. Nielsen, A. Friis-Møller, H. Fjeldsøe-Nielsen, and K. Schønning, “Prevalence and molecular characterization of clinical isolates of *Escherichia coli* expressing an AmpC phenotype.,” *J. Antimicrob. Chemother.*, vol. 65, no. 3, pp. 460–4, Mar. 2010.

[109] L. Poirel, T. R. Walsh, V. Cuvillier, and P. Nordmann, “Multiplex PCR for detection of acquired carbapenemase genes.,” *Diagn. Microbiol. Infect. Dis.*, vol. 70, no. 1, pp. 119–23, May 2011.

[110] I. Wiegand, H. K. Geiss, D. Mack, E. Stürenburg, and H. Seifert, “Detection of extended-spectrum beta-lactamases among Enterobacteriaceae by use of semiautomated microbiology systems and manual detection procedures.,” *J. Clin. Microbiol.*, vol. 45, no. 4, pp. 1167–74, Apr. 2007.

[111] G. Arlet, M. Rouveau, and A. Philippon, “Substitution of alanine for aspartate at position 179 in the SHV-6 extended-spectrum beta-lactamase.,” *FEMS Microbiol. Lett.*, vol. 152, no. 1, pp. 163–7, Jul. 1997.

[112] B. Bedenić, C. C. Randegger, E. Stobberingh, and H. Hächler, “Molecular epidemiology of extended-spectrum beta-lactamases from *Klebsiella pneumoniae* strains isolated in Zagreb, Croatia.,” *Eur. J. Clin. Microbiol. Infect. Dis.*, vol. 20, no. 7, pp. 505–8, Jul. 2001.

[113] I. Olesen, H. Hasman, and F. M. Aarestrup, “Prevalence of beta-lactamases among ampicillin-resistant *Escherichia coli* and *Salmonella* isolated from food animals in Denmark.,” *Microb. Drug Resist.*, vol. 10, no. 4, pp. 334–40, Jan. 2004.

[114] M. T. Nüesch-Inderbinen, H. Hächler, and F. H. Kayser, “Detection of genes coding for extended-spectrum SHV beta-lactamases in clinical isolates by a molecular genetic method, and comparison with the E test.,” *Eur. J. Clin. Microbiol. Infect. Dis.*, vol. 15, no. 5, pp. 398–402, May 1996.

[115] “The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 5.0,” 2015. [Online]. Available: http://www.eucast.org.

[116] W.-L. Yu, W.-C. Ko, K.-C. Cheng, H.-C. Lee, D.-S. Ke, C.-C. Lee, C.-P. Fung, and Y.-C. Chuang, “Association between *rmpA* and *magA* genes and clinical syndromes caused by *Klebsiella pneumoniae* in Taiwan.,” *Clin. Infect. Dis.*, vol. 42, no. 10, pp. 1351–8, May 2006.

[117] R. Podschun, A. Fischer, and U. Ullman, “Expression of putative virulence factors by clinical isolates of *Klebsiella planticola*.,” *J. Med. Microbiol.*, vol. 49, no. 2, pp. 115–9, Feb. 2000.

[118] M. A. Schembri, J. Blom, K. A. Krogfelt, and P. Klemm, “Capsule and fimbria interaction in *Klebsiella pneumoniae*.,” *Infect. Immun.*, vol. 73, no. 8, pp. 4626–33, Aug. 2005.

[119] J. H. Merritt, D. E. Kadouri, and G. A. O’Toole, “Growing and analyzing static biofilms.,” *Curr. Protoc. Microbiol.*, vol. Chapter 1, p. Unit 1B.1, Jul. 2005.

[120] T. A. Oelschlaeger, D. Zhang, S. Schubert, E. Carniel, W. Rabsch, H. Karch, and J. Hacker, “The high-pathogenicity island is absent in human pathogens of *Salmonella enterica* subspecies I but present in isolates of subspecies III and VI.,” *J. Bacteriol.*, vol. 185, no. 3, pp. 1107–11, Feb. 2003.

[121] W.-L. Yu, C.-P. Fung, W.-C. Ko, K.-C. Cheng, C.-C. Lee, and Y.-C. Chuang, “Polymerase chain reaction analysis for detecting capsule serotypes K1 and K2 of *Klebsiella pneumoniae* causing abscesses of the liver and other sites.,” *J. Infect. Dis.*, vol. 195, no. 8, pp. 1235–6; author reply 1236, Apr. 2007.

[122] V. Donati, F. Feltrin, R. S. Hendriksen, C. A. Svendsen, G. Cordaro, A. García-Fernández, S. Lorenzetti, R. Lorenzetti, A. Battisti, and A. Franco, “Extended-spectrum-beta-lactamases, AmpC beta-lactamases and plasmid mediated quinolone resistance in *Klebsiella* spp. from companion animals in Italy.,” *PLoS One*, vol. 9, no. 3, p. e90564, Jan. 2014.

[123] M. Y. Lee, K. S. Ko, C.-I. Kang, D. R. Chung, K. R. Peck, and J.-H. Song, “High prevalence of CTX-M-15-producing *Klebsiella pneumoniae* isolates in Asian countries: diverse clones and clonal dissemination.,” *Int. J. Antimicrob. Agents*, vol. 38, no. 2, pp. 160–3, Aug. 2011.

[124] J. B. Nielsen, M. N. Skov, R. L. Jørgensen, O. Heltberg, D. S. Hansen, and K. Schønning, “Identification of CTX-M15-, SHV-28-producing *Klebsiella pneumoniae* ST15 as an epidemic clone in the Copenhagen area using a semi-automated Rep-PCR typing assay.,” *Eur. J. Clin. Microbiol. Infect. Dis.*, vol. 30, no. 6, pp. 773–8, Jun. 2011.

[125] C. Rodrigues, E. Machado, H. Ramos, L. Peixe, and Â. Novais, “Expansion of ESBL-producing *Klebsiella pneumoniae* in hospitalized patients: a successful story of international clones (ST15, ST147, ST336) and epidemic plasmids (IncR, IncFIIK).,” *Int. J. Med. Microbiol.*, vol. 304, no. 8, pp. 1100–8, Nov. 2014.

[126] C. Ewers, I. Stamm, Y. Pfeifer, L. H. Wieler, P. A. Kopp, K. Schønning, E. Prenger-Berninghoff, S. Scheufen, I. Stolle, S. Günther, and A. Bethe, “Clonal spread of highly successful ST15-CTX-M-15 *Klebsiella pneumoniae* in companion animals and horses.,” *J. Antimicrob. Chemother.*, vol. 69, no. 10, pp. 2676–80, Oct. 2014.

[127] D. Quiñones, A. Valverde, M. Rodríguez-Baños, N. Kobayashi, A. Zayaz, M. Abreu, R. Cantón, and R. del Campo, “High clonal diversity in a non-outbreak situation of clinical ESBL-producing *Klebsiella pneumoniae* isolates in the first national surveillance program in Cuba.,” *Microb. Drug Resist.*, vol. 20, no. 1, pp. 45–51, Feb. 2014.

[128] G. Marcade, S. Brisse, S. Bialek, E. Marcon, V. Leflon-Guibout, V. Passet, R. Moreau, and M.-H. Nicolas-Chanoine, “The emergence of multidrug-resistant *Klebsiella pneumoniae* of international clones ST13, ST16, ST35, ST48 and ST101 in a teaching hospital in the Paris region.,” *Epidemiol. Infect.*, vol. 141, no. 8, pp. 1705–12, Aug. 2013.

[129] A. Poulou, E. Voulgari, G. Vrioni, V. Koumaki, G. Xidopoulos, V. Chatzipantazi, F. Markou, and A. Tsakris, “Outbreak caused by an ertapenem-resistant, CTX-M-15-producing *Klebsiella pneumoniae* sequence type 101 clone carrying an OmpK36 porin variant.,” *J. Clin. Microbiol.*, vol. 51, no. 10, pp. 3176–82, Oct. 2013.

[130] A. Tóth, B. Kocsis, I. Damjanova, K. Kristóf, L. Jánvári, J. Pászti, R. Csercsik, J. Topf, D. Szabó, P. Hamar, K. Nagy, and M. Füzi, “Fitness cost associated with resistance to fluoroquinolones is diverse across clones of *Klebsiella pneumoniae* and may select for CTX-M-15 type extended-spectrum β-lactamase.,” *Eur. J. Clin. Microbiol. Infect. Dis.*, vol. 33, no. 5, pp. 837–43, May 2014.

[131] H. Sahly, S. Navon-Venezia, L. Roesler, A. Hay, Y. Carmeli, R. Podschun, C. Hennequin, C. Forestier, and I. Ofek, “Extended-spectrum beta-lactamase production is associated with an increase in cell invasion and expression of fimbrial adhesins in *Klebsiella pneumoniae*.,” *Antimicrob. Agents Chemother.*, vol. 52, no. 9, pp. 3029–34, Sep. 2008.

[132] H. Sahly, H. Aucken, V. J. Benedí, C. Forestier, V. Fussing, D. S. Hansen, I. Ofek, R. Podschun, D. Sirot, J. M. Tomás, D. Sandvang, and U. Ullmann, “Increased serum resistance in *Klebsiella pneumoniae* strains producing extended-spectrum beta-lactamases.,” *Antimicrob. Agents Chemother.*, vol. 48, no. 9, pp. 3477–82, Sep. 2004.

[133] P. Di Martino, D. Sirot, B. Joly, C. Rich, and A. Darfeuille-Michaud, “Relationship between adhesion to intestinal Caco-2 cells and multidrug resistance in *Klebsiella pneumoniae* clinical isolates.,” *J. Clin. Microbiol.*, vol. 35, no. 6, pp. 1499–503, Jun. 1997.

[134] P. Di Martino, V. Livrelli, D. Sirot, B. Joly, and A. Darfeuille-Michaud, “A new fimbrial antigen harbored by CAZ-5/SHV-4-producing *Klebsiella pneumoniae* strains involved in nosocomial infections.,” *Infect. Immun.*, vol. 64, no. 6, pp. 2266–73, Jun. 1996.

[135] J. Shin and K. S. Ko, “Comparative study of genotype and virulence in CTX-M-producing and non-extended-spectrum-β-lactamase-producing *Klebsiella pneumoniae* isolates.,” *Antimicrob. Agents Chemother.*, vol. 58, no. 4, pp. 2463–7, Jan. 2014.

[136] J. Shin, D. H. Kim, and K. S. Ko, “Comparison of CTX-M-14- and CTX-M-15-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates from patients with bacteremia.,” *J. Infect.*, vol. 63, no. 1, pp. 39–47, Jul. 2011.

[137] R. Leclercq, R. Cantón, D. F. J. Brown, C. G. Giske, P. Heisig, A. P. MacGowan, J. W. Mouton, P. Nordmann, A. C. Rodloff, G. M. Rossolini, C.-J. Soussy, M. Steinbakk, T. G. Winstanley, and G. Kahlmeter, "EUCAST expert rules in antimicrobial susceptibility testing.," *Clin. Microbiol. Infect.*, vol. 19, no. 2, pp. 141–60, Feb. 2013.

[138] B. Libisch, C. G. Giske, B. Kovács, T. G. Tóth, and M. Füzi, "Identification of the first VIM metallo-beta-lactamase-producing multiresistant *Aeromonas hydrophila* strain.," *J. Clin. Microbiol.*, vol. 46, no. 5, pp. 1878–80, May 2008.

[139] B. Libisch, M. Muzslay, M. Gacs, J. Minárovits, M. Knausz, J. Watine, G. Ternák, E. Kenéz, I. Kustos, L. Rókusz, K. Széles, B. Balogh, and M. Füzi, "Molecular epidemiology of VIM-4 metallo-beta-lactamase-producing *Pseudomonas* sp. isolates in Hungary.," *Antimicrob. Agents Chemother.*, vol. 50, no. 12, pp. 4220–3, Dec. 2006.

[140] C. Arpin, P. Noury, D. Boraud, L. Coulange, A. Manetti, C. André, F. M'Zali, and C. Quentin, "NDM-1-producing *Klebsiella pneumoniae* resistant to colistin in a French community patient without history of foreign travel.," *Antimicrob. Agents Chemother.*, vol. 56, no. 6, pp. 3432–4, Jun. 2012.

[141] L. Poirel, A. Benouda, C. Hays, and P. Nordmann, "Emergence of NDM-1-producing *Klebsiella pneumoniae* in Morocco.," *J. Antimicrob. Chemother.*, vol. 66, no. 12, pp. 2781–3, Dec. 2011.

[142] T. Netikul, H. E. Sidjabat, D. L. Paterson, W. Kamolvit, W. Tantisiriwat, J. A. Steen, and P. Kiratisin, "Characterization of an IncN2-type *bla*NDM-$_1$-carrying plasmid in *Escherichia coli* ST131 and *Klebsiella pneumoniae* ST11 and ST15 isolates in Thailand.," *J. Antimicrob. Chemother.*, vol. 69, no. 11, pp. 3161–3, Nov. 2014.

[143] G. Peirano, J. Ahmed-Bentley, J. Fuller, J. E. Rubin, and J. D. D. Pitout, "Travel-related carbapenemase-producing Gram-negative bacteria in Alberta, Canada: the first 3 years.," *J. Clin. Microbiol.*, vol. 52, no. 5, pp. 1575–81, May 2014.

[144] D. M. Arana, D. Saez, P. García-Hierro, V. Bautista, S. Fernández-Romero, M. Ángel de la Cal, J. I. Alós, and J. Oteo, "Concurrent interspecies and clonal dissemination of OXA-48 carbapenemase.," *Clin. Microbiol. Infect.*, vol. 21, no. 2, pp. 148.e1–4, Oct. 2014.

[145] M. Österblad, J. Kirveskari, A. J. Hakanen, P. Tissari, M. Vaara, and J. Jalava, "Carbapenemase-producing Enterobacteriaceae in Finland: the first years (2008-11).," *J. Antimicrob. Chemother.*, vol. 67, no. 12, pp. 2860–4, Dec. 2012.

[146] E. Liapis, A. Pantel, J. Robert, M.-H. Nicolas-Chanoine, L. Cavalié, N. van der Mee-Marquet, C. de Champs, N. Aissa, C. Eloy, V. Blanc, C. Guyeux, D. Hocquet, J.-P. Lavigne, and X. Bertrand, “Molecular epidemiology of OXA-48-producing *Klebsiella pneumoniae* in France.,” *Clin. Microbiol. Infect.*, Jun. 2014.

[147] I. Sánchez-Romero, A. Asensio, J. Oteo, M. Muñoz-Algarra, B. Isidoro, A. Vindel, J. Alvarez-Avello, B. Balandín-Moreno, O. Cuevas, S. Fernández-Romero, L. Azañedo, D. Sáez, and J. Campos, “Nosocomial outbreak of VIM-1-producing *Klebsiella pneumoniae* isolates of multilocus sequence type 15: molecular basis, clinical risk factors, and outcome.,” *Antimicrob. Agents Chemother.*, vol. 56, no. 1, pp. 420–7, Jan. 2012.

[148] C. Rodrigues, Â. Novais, E. Machado, and L. Peixe, “Detection of VIM-34, a novel VIM-1 variant identified in the intercontinental ST15 *Klebsiella pneumoniae* clone.,” *J. Antimicrob. Chemother.*, vol. 69, no. 1, pp. 274–5, Jan. 2014.

[149] D. L. Veras, L. C. Alves, F. A. Brayner, D. R. D. Guedes, M. A. V. Maciel, C. R. C. Rocha, and A. C. de Souza Lopes, “Prevalence of the *bla*(SHV) gene in *Klebsiella pneumoniae* isolates obtained from hospital and community infections and from the microbiota of healthy individuals in Recife, Brazil.,” *Curr. Microbiol.*, vol. 62, no. 5, pp. 1610–6, May 2011.

[150] “EUCAST Frequently Asked Questions.” [Online]. Available: www.eucast.org. [Accessed: 22-Jun-2015].

[151] P. J. Bergen, C. B. Landersdorfer, J. Zhang, M. Zhao, H. J. Lee, R. L. Nation, and J. Li, “Pharmacokinetics and pharmacodynamics of ‘old’ polymyxins: what is new?,” *Diagn. Microbiol. Infect. Dis.*, vol. 74, no. 3, pp. 213–23, Nov. 2012.